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13. ABSTRACT ***(Maximum 200 Words)***

Mechanical stimulation is crucial to the homeostasis of adult bone density and mass. The hypothesis of this proposal is that bone cells sense their mechanical environment through specific cell surface receptors (integrins) that interact with specific extracellular matrix (ECM) proteins (osteopontin, bone sialoprotein, and fibronectin) that are the ligands for these receptors. We propose that the expression of these proteins is regulated in response to both cellular interactions with the ECM and mechanical stimulation. Thus, these proteins act like autocrine factors that modify cell behavior in response to changes in either matrix composition or mechanical deformation of the ECM itself. The proposed experiments will define how osteoblasts discriminate the molecular mechanisms by which mechanical signals mediate their actions through the cellular interactions of integrins with the ECM. A determination of the specific integrin isotypes that are involved in the mechano-signal transduction process will be made. The signal transduction system(s) that are responsible for mediating osteopontin, bone sialoprotein and fibronectin gene expression in response to mechanical stimulation, will be determined. Other experiments will examine how aspects of the mechanical stimuli, such as frequency, intensity or duration, effect cell response. Knowledge gained from understanding mechano-signal transduction will facilitate the development of appropriate clinical approaches to enhance the adaptive responses of skeletal tissue to mechanical stimulation.

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Table of Contents

Face Page Attachment 1 1

SF298 2

Table of Contents.....3

Introduction 4

Body 4-9

Conclusions 9-10

References.....10-11

Institutional letters of compliance for use of
biohazards, radiation and animals.....12- 14

Appendices: 15

A. Selective Induction of OPN expression in Osteoblasts is Mediated by Different Integrin Ligands. (Abstract)

B. The Predominant Integrin Ligands Expressed by Osteoblasts Show Preferential Regulation in Response to both Cell Adhesion and Mechanical Perturbation. (Manuscript)

C. Differential Induction of Osteopontin Gene Expression by Osteoblasts in Response to Adhesion to Selective Integrin Ligands. (Manuscript)

D. Induction of a Neoarthrosis by Precisely Controlled Motion in an Experimental Mid-Femoral Defect. (Manuscript)

INTRODUCTION

The bone remodeling cycle is known to be intimately involved in the metabolic homeostasis of mineral balance.[1] Bone formation and the remodeling cycle have been shown to be essential in maintaining the structural integrity of skeletal tissue in response to the mechanical loading to which it is subjected.[2,3] Bone remodeling has also been hypothesized to provide the means of repairing bone tissue that has been damaged as a result of mechanical fatigue.[4] Thus, it may be speculated that the skeletal cells (osteoblasts and osteoclasts) which mediate the remodeling process are regulated by their mechanical environment. In order for osteoblasts to respond to their mechanical environment, they must in some way sense it. One mechanism by which cells sense mechanical signals is through the physical deformation of the tissue. The hypothesis of this proposal was that specific cell surface receptors (integrins) that interact with specific extracellular matrix proteins (collagen, osteopontin, bone sialoprotein, and fibronectin) provide the physical link through which mechanical stimuli are transmitted via tissue deformation. As a corollary to this hypothesis, we proposed that the extracellular proteins that are ligands for these receptors are themselves regulated in response to cellular interaction with both the matrix and mechanical stimulation. Thus, these proteins act like autocrine factors that modify cell behavior in response to changes in matrix composition and mechanical deformation, as well as having a structural role in the matrix itself. Maintaining the balance of the extracellular matrix composition within bone then provides a mechanism by which the structural homeostasis of the skeleton may be regulated. This hypothesis and the published data that supports it are found in our review article published in 1999.[5]

The experiments that were carried out with the support of this grant were designed to define how osteoblasts and skeletal cells discriminate mechanical stimuli at a molecular level and how mechanical stimuli and cellular interactions with the extracellular matrix are transduced through integrin receptors and converted to intracellular signals that lead to genomic changes. These experiments defined whether different integrin ligands use common mechanisms in the regulation of their response to these stimuli. A determination of the intracellular second signaling systems (kinases) that are responsible for mediating the altered gene expression of *opn*, *bsp* and *fn* to mechanical stimulation were determined, both by assessing which kinases are activated and through the use of specific inhibitors that block the actions of the different kinase systems. A determination of the specific integrin isotypes that are involved in the different signal transduction processes was made. Other experiments examined if individual gene responses are differentially sensitive to various aspects of the mechanical stimuli (intensity, frequency, duration) or have different thresholds of response to different component parts of the stimuli.

BODY (with Goals as Defined in the Original Proposal)

Goal 1 The first goal of this proposal was directed at defining the molecular mechanisms of signal transduction by which mechanical stimulation regulates the expression of three specific ECM genes (osteopontin, bone sialoprotein and fibronectin) within osteoblasts. These studies tested whether there are common signal transduction pathways that mediate changes in the expression of these genes in response to mechanical stimulation.

a) The involvement of specific integrin isotypes in the mechano-signal transduction process that mediates these gene's genomic responses to mechanical stimulation was assessed in year 1.
b) The mechanistic relationship between the signal transduction pathways that are activated by mechanical stimulation vs. cell adhesion was examined in years 2-3
c) The nature of the second signal transduction pathways that mediate the changes in expression for each of these genes was determined in years 2-3.

Results of Goal 1

The effect of cell adhesion on the expression of these genes was separately examined to determine if this stimuli would mimic the effects of the mechanical stimulation. All three genes showed comparable levels of induction in response to adhesion on the fibronectin coated surfaces in comparison to non-coated surfaces, with maximal levels of induction seen for *bsp* and *opn* at 24 hours after plating while *fn* showed maximal levels of stimulation at 8 hours. Interestingly, while both *opn* and *fn* mRNA expression returned to base line after cell adhesion on fibronectin, *bsp* mRNA levels remained elevated. Examination of the signal transduction pathways that mediated the gene expression in response to attachment on fibronectin coated surfaces showed that both genistein and cycloheximide inhibited the induction of all three genes. This demonstrates that a tyrosine kinase was involved in the cell attachment mediated induction of these genes, and new protein synthesis was a prerequisite to this process. In contrast, the PKA specific inhibitor H-89 only ablated the induction of *fn* expression. Depolymerization of either microtubules or microfilaments with colchicine or cytochalasin D respectively had little effect on the over all expression of these genes in response to cell adhesion, indicating that the adhesion mediated phenomena was not dependent on cytoskeletal integrity. In summary, these results show that both mechanical stimulation and cell adhesion specifically stimulated the expression of integrin binding proteins. These results further demonstrate that while there are common features in the signal transduction processes that mediated the induction of these genes, each gene was separately induced by unique mechanisms. **A complete description of these results is in a manuscript that has been accepted for publication.** Carvalho RS, Schaffer JL, Bumann A and Gerstenfeld LC (2001) *"RGD containing proteins of osteoblasts are responsive to mechanical stimulation and matrix attachment."* See appended materials.

In our second study, we examined three interrelated relationships pertaining to osteoblast adhesion and the transduction of molecular signals. This study is described completely in appended material and in part below. The first aspect of this study was to define if osteoblasts have variable adhesion properties to different integrin ligands. The second component of the study was to define the relationship between the selective adhesion of the osteoblasts on the different ligands with the induction of specific second signal kinase activities. The final aspect of this study was to determine if osteoblast adhesion to specific integrin ligands would show specific induction of *opn* mRNA expression. The first series of experiments of this study are depicted in Figure 1, panels A and B. These data show that three different extracellular matrix proteins, fibronectin (FN), fibrillar collagen type I (C), and denatured collagen type I gelatin (G), promote selective adhesion, whereas three other proteins, laminin (LN), osteopontin (OPN), and vitronectin (VN), did not. Bovine serum albumin (Al) was used as a control and is included in Panel B. As can be seen from this study, fibronectin was the most effective protein at promoting selective adhesion. Both native and denatured type I collagen also promoted selective

attachment, but unlike fibronectin, both native and denatured collagen showed saturation at between 1 and 3μg/cm^2 of surface coating. In contrast, fibronectin did not show saturation in promoting cell adhesion until about 30μg/cm^2 (data not shown). These data demonstrate that osteoblast adhesion specifically led to a strong induction of p-tyrosine levels and a generalization of its distribution throughout the cells after adhesion was promoted with a specific integrin binding ligand. These data then reinforce the conclusions reached in the experiments depicted in Figure 2, which suggest that the induction of intracellular kinase activities are related more to the specific nature of the ligand's interactions with its receptor and less with the process of cellular adhesion alone.

The last set of experiments of this study examined the relationship between osteoblast adhesion on specific ligands and the induction of specific genomic changes, using *opn* mRNA levels as a marker of genomic regulation. These studies are depicted in Figure 4. As can be seen in this figure, the induction of increased *opn* mRNA expression was seen as early as four hours after the cells had been allowed to adhere and continued to increase up to 24 hours. It is interesting to note in this study that once again native fibrillar collagen, fibronectin (*fn*), and to a lesser extent laminin, induced osteopontin (*opn*) gene expression to increase. These results suggest that the same integrin receptors that facilitate specific cell attachment also facilitate the induction of osteopontin expression. It is also interesting to note that neither osteopontin nor vitronectin mediated either of these cell responses, suggesting that they are not facilitated through a $\alpha_v\beta_3$ receptor. The one difference that was observed, however, was in the comparison of cell attachment on denatured collagen versus the induction of osteopontin. This result again suggests that signal transduction through the collagen receptor is only mediated when it interacts with native collagen. **A complete description of these results is in a manuscript that is being prepared for publication.** Carvalho RS, Kostenuick P, Bumann A, Salih E and Gerstenfeld LC (2001) *"Osteoblast adhesion to different integrin ligands selectively mediates osteopontin gene expression."* (Manuscript in preparation) See appended materials.

Goal 2 The second goal of these studies determined the relative importance of each component part of the mechanical stimuli (intensity, frequency and duration) to the mechano-signal transduction process.

a) The components of the mechanical stimuli (intensity, frequency, and duration) that is responsible for producing the signal transduction, which leads to the genomic regulation of specific genes in osteoblasts, were examined in year 3.
b) Short term (<24hr) versus chronic (>72hr) adaptive responses of osteoblasts to variations in duration of mechanical perturbation were defined in year 3.

Results of Goal 2

Towards the completion of goal two, the first series of these experiments was carried out by Emily Samuels, a masters student with the Harvard School of Dental Medicine, and the repeat of these studies was carried out by Anagret Rebin, a masters student from the Dental School of University of Kiel, Germany (on sabbatical). These experiments were focused to determine the component part of mechanical stimuli that would induce changes in osteopontin gene expression. In both sets of studies we analyzed duration and frequency of the mechanical stimulation and examined the effect of fluid flow-induced shear stress on osteopontin expression. Using our

device, which delivers spatially uniform biaxial strain to a membrane surface, osteoblasts were subjected to 1% strain for 1, 5, 15 and 30 minutes and 1, 2, 4 and 8 hours of strain. Analysis of the induction of osteopontin mRNA expression demonstrated that a maximal 145% induction was observed after 8 hours. ($p<0.05$). Osteoblasts were then subjected to varying frequencies of strain (0.1, 0.25, 0.5 and 1 Hz) for 8 hours. There was clearly a 2-3 fold stimulation in response to increasing frequency again with a significant finding ($p<0.05$). The effect of fluid induced shear stress was examined in the third part of this study. This was accomplished in the following manner. The membrane was cut into two equal areas that encompassed the inner and outer circular areas of the membrane. The cells were then subjected to an 8 hour period of mechanical stimulation at the same variations in frequency as used in the first part of these studies. Because of the way the device generates its strains, the inner circular area has several fold lower fluid flow than the outer areas. Similarly, higher frequencies will generate higher fluid flows; thus the combination of inner and outer areas with increasing frequency of strain should produce several orders of magnitude greater fluid movement. Analysis of *opn* mRNA production, however, showed that only the highest frequency (1 Hz) generated altered levels of osteopontin expression when comparing the inner and outer areas of the membrane. These results also showed that it was inversely related to the levels of fluid flow. Thus, the inner areas had the higher levels of osteopontin induction. Because a second student repeated these experiments and obtained the same results, we currently believe that the initial results that we obtained are correct. These results, however, are completely opposite to the hypothesis that fluid shear stress was the major mechanical stimulation that facilitated osteopontin (*opn*) expression. These results also would lead us questions as to whether Ca flux through stress activated channels was a component part of the signal transduction mechanisms that induced osteopontin expression as has been suggested by other research groups. This later suggestion, however, has since been shown to be incorrect.[7] As we moved forward with the third year and finished out these studies, we assessed how to further examine the mechanisms by which strain is effecting osteopontin gene regulation by determining the arrangement of stress fibers and integrin receptor localization within the cells that are found within the various areas of the mechanically stimulated membranes. These studies completed goal two and we anticipate publishing one more manuscript of these our findings.

Continuation of Ongoing and New Studies

New studies that are ongoing and were derived from funding from this application are now directed towards the future long-term goals of extrapolating these model studies into a mammalian system and examining the effects of mechanical stimulation on skeletal stem cell differentiation.

Preliminary mechanical stimulation experiments have been undertaken in C3H10T1/2 murine mesenchymal stem cells and mesenchymal stem cells isolated from murine facture calluses at 7 days when the tissue is predominantly cartilage. These new experiments have the goal of determining how these cells respond to mechanical stimulation and if mechanical stimulation will change the time course of differentiation that is induced by BMP-7 treatment. Initial experiments will focus on examining osteopontin. Further experiments will assess the markers of both osteogenic and chondrogenic differentiation to determine if mechanical stimulation promotes chondrogenic or osteogenic differentiation selectively. In conjunction with

these in vitro studies, we have established an in vivo model of introducing exact defined micromotion into an osteotomy gap. These experiments are described in *"Induction of a neoarthrosis by precisely controlled motion in an experimental mid-femoral defect,"* which was published in the Journal of Orthopaedic Research and is included in the appended materials.

KEY RESEARCH ACCOMPLISHMENTS

1. We have completed all of the experiments outlined in goal one.

2. We have completed the components of goal two and are currently repeating a number of experiments that we initiated in year one.

3. Beyond the listed publications, we will publish one to two more manuscripts from work supported by these studies.

4. We established a new model of in vivo mechanical stimulation to study the role of mechanical forces in promoting chondrogenic differentiation in an osteotomy gap in a rat model.

Reportable Outcomes 2000/2001
Manuscripts, Abstracts, and Presentations

Manuscripts:
a) Carvalho RS, Schaffer JL, Bumann A and Gerstenfeld LC. (2001) The predominant integrin ligands expressed by osteoblasts show preferential regulation in response to both cell adhesion and mechanical stimulation. J. Cellular Biochem. (in Press)

b) Cullinane DM, Frederick A, Eisenberg SR, Paccica D, Elaman MV, Lee C, Salisbury K, Gerstenfeld LC and Einhorn T. (2001) Induction of neoarthrosis by precisely controlled motion in an experimental mid-femoral defect. J. Orthopaedic Res. (in Press)

c) Carvalho RS. Kostenuick P, Bumann A, Salih E and Gerstenfeld LC. (2001) Osteoblast adhesion to different integrin ligands selectively mediates osteopontin gene expression. (Manuscript in preparation) (appended in preliminary form)

Abstracts:
Carvalho RS. Kostenuick P, Bumann A, Salih E and Gerstenfeld LC (2001) Selective induction of osteopontin expression in osteoblasts is mediated by different integrin ligands. J. Bone Mineral Res. 16:S1:S:240.

Carvalho RS, Kostenuick P, Bumann A and Gerstenfeld LC. (2002) Differential Mechanisms of Signal Transduction that Mediate the Induction of Gene Expression by Cell Adhesion or Mechanical Stimulation within Osteoblasts. Orthopaedic Research Society.

Presentations:
Cullinane D, Frederick A, Gerstenfeld L, Eisenberg S, Pacicca D, Altuwairgi O, Lee C, Salisbury K, Hellenbrand L and Einhorn T (2001) Induction of joint morphology and articular-like cartialge in a bone gap using controlled micromotion. Transactions of Ortho Res. Soc, 26:364

Patents: None

Degrees obtained supported by this award: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models, etc:
New Animal Model: Dr. Dennis Cullinane, who received partial support from this application, developed a new research area related to mechanical stimulation in bone repair. This new research project is focused on a new model of bone repair in which an external fixator is used to apply controlled micro motion to an osteotomy. Using this surgical approach new joint like structures are able to be formed in a rat model. This approach may have clinical applications for use in the reconstruction of finger joints. This new animal model is now being applied to study cartilage formation.

Funding applied for based on work supported by this award:
a) Arthritis Foundation; Dennis M. Cullinane Ph.D.
b) Continuation of funding for this project will be applied for from the NIH in 2002

Employment or research opportunities applied for and/or received on experiences/training supported by this award:
a) In the last year two M.D./Ph.D. candidates have been recruited into this laboratory to work on areas of biomechanical stimulation of bone healing and bone cell response. Ms. Kristy Salisbury, a bioengineering student, will be working on the new project area of joint regeneration by biomechanical stimulation. Mr. Alexios Apazidis, a biochemistry student, will be continuing work using cultured cells and mechanical stimulation. In the coming year we will be assessing effects of mechanical stimulation on mesenchymal stem cells and cultured cells from fracture callus tissues as we rewrite this project for NIH funding.

CONCLUSIONS

The major conclusions from this proposal are as follows:

1. Integrin ligands as a class are induced in osteoblasts in response to mechanical stimulation and adhesion. Such findings provide strong evidence to support the hypothesis that these molecules act like autocrine or paracrine factors.

2. Cell adhesion of osteoblasts is specifically mediated by β1 class of integrins. This same class of integrins appears to be responsible for the signal transduction process that stimulates osteopontin induction. The β3 integrin ligands vitronectin and osteopontin neither mediate

specific adhesion nor induce osteopontin gene expression, but they do selectively induce specific kinase activities.

3. Component aspects of the mechanical stimulation do affect the induction of the gene. Both greater duration and increasing frequency clearly appear to enhance response to the mechanical signal. Shear stress induced by fluid flow does not appear to be a mediating factor in osteopontin induction in response to mechanical stimulation.

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Abstracts:

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Carvalho RS, Kostenuick P, Bumann A and Gerstenfeld LC. (2002) Differential Mechanisms of Signal Transduction that Mediate the Induction of Gene Expression by Cell Adhesion or Mechanical Stimulation within Osteoblasts. Orthopaedic Research Society.

Presentations :

Differential Mechanisms of Signal Transduction that Mediate the Induction of Gene Expression by Cell Adhesion or Mechanical Stimulation within Osteoblasts. Presented at "Osteoporosis as a

failure of bone's adaptation to functional load bearing." The Wellecome Trust Foundation, Highgate, England 1999.

Final List of Personnel:

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Michael Elman, B.S.
Erjan Salih, Ph.D. (Subcontract)

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BOSTON UNIVERSITY MEDICAL CENTER

Radiation Protection Office

88 East Newton Street, D-604
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To: Dr. Gerstenfeld

Date: June 24, 1998

From: Victor Evdokimoff, Secretary Radioisotope Committee VNE/Cm

Subject: Authorization to use radioisotopes at BUMC

On June 24, 1998 your application _X_, renewal ___, amendment___ to use radioisotopes at BUMC was approved. You are only authorized for the following isotope(s), quantities, etc.

Isotope(s)	Form	Max./order	Max./year	Possession limit
H-3	Amino acid, Nucleotide	5 mCi	N/A	N/A
P-32	Phosphorous, Nucleotide	5 mCi	N/A	N/A
C-14	Chloramphenicol, Amino acids	1 mCi	N/A	N/A
S-35	Na Sulfate, Nucleotide, Amino acids	1 mCi	N/A	N/A
P-33	Phosphorous, Nucleotides	1 mCi	N/A	N/A

Your authorization code number is G-16 . This number must appear on all requisitions for isotopes. In addition, the following **conditions** apply to your authorization:

1) The Radioisotope Committee is recommending you consider alternatives to using 3H/14C such as non-radioactive tracers.

2) All personnel under your permit planning on using P-32 must complete the individual training requirements. In addition, any user of 1 mCi or more of P-32 at one time is required to be monitored by TLD. Please contact the RPO for assistance.

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Medical Campus

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BOSTON UNIVERSITY MEDICAL CENTER

Institutional Biosafety Committee

Renewal Letter: Biohazard Project **IBC Coordinator:** Mary Gistis

Principal Investigator:	Dr. Louis Gerstenfeld
Project Title:	Musculoskeletal Research
Approval Number:	# A-168
Renewal Date:	November 4, 1998
Containment Level:	BL-2/Universal Precautions
Comments:	related to rDNA projects 497, 500, 501

For your records, make several copies of this document to avoid delays when filing your grant applications with federal agencies.

IBC APPROVAL LETTER

Boston University
School of Medicine

Institutional Animal
Care and Use
Committee

700 Albany Street, W707
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Louis C. Gerstenfeld PhD
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7/11/2000

RE: Application No. 98-096 Agency: Department of the Army

Title: Mechanisms of Mechano-Transduction Within Osteoblasts

Protocol Status: APPROVED, 7/22/1998
ANIMAL NUMBERS/YR. 1248 chicken embryos/year x 4 yrs

BIOHAZARDS:

Dear Dr. Gerstenfeld

Your application for use of animals in research or education has been reviewed by the Institutional Animal Care and Use Committee at Boston University Medical Center. The protocol is APPROVED as being consistent with humane treatmen of laboratory animals and with standards set forth in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act.

The Laboratory Animal Science Center at Boston University Medical Center has been accredited by the American Association for Accreditation of Laboratory Animal Care since 1971. Boston University Medical Center has had an Animal Welfare Assurance on file with the Office for Protection from Research Risks (OPRR) since January 1, 1986. Boston University's Animal Welfare Assurance number is A-3316-01.

Animal protocols may be approved for up to three years. However, if the study extends beyond one year from the approval date, an annual continuation form (1 page) must be submitted. If a project is to extend beyond three years, a full application must be resubmitted and reviewed at the end of the initial three year period.

Sincerely,

Colleen A. Cody, Coordinator
Institutional Animal Care and Use Committee

c: Wayne W. LaMorte, M.D., Ph.D., M.P.H. Chairman, IACUC
Veterinary Staff, LASC

Appendix A

"Selective Induction of OPN Expression in Osteoblasts is Mediated by Different Integrin Ligands"
R.S. Carvalho, P.J. Kostenuik, A. Bumann, E. Salih, L.C. Gerstenfeld

Journal of Bone and Mineral Research, Vol 16, Suppl 1, Sept 2001, pp S240
Abstract SA052

Final Report

DAMD17-98-1-8510

Bone-97 Mechanisms of Mechano-Transduction within Osteoblasts

P.I., Louis C. Gerstenfeld, Ph.D.

Selective Induction of OPN Expression in Osteoblasts is Mediated by Different Integrin Ligands. R. S. Carvalho[*1], P.J. Kostenuik[*2], A. Bumann[*3], E. Salih[*4], L.C. Gerstenfeld[1]
1Orthopaedic Surgery, Boston University Medical Center, Boston, MA, USA. [2]Amgen, Thousand Oaks, CA, USA. [3]University of Southern California, Los Angeles, CA, USA. [4]Children's Hospital, Boston, MA, USA.

Bone remodeling is partly regulated by the mechanical environment of skeletal cells. However, some forms of signals derived from mechanical stimulation are only perceived by cells after adhesion has taken place. Therefore, signal transduction and gene expression events activated by the latter may be similar, if not identical, to those induced by integrin mediated cell adhesion. Recent studies have shown that osteopontin (OPN) mediates osteoblastic and osteoclastic interaction with the mineralized matrix. Thus, OPN is both associated with remodeling and formation. These studies examined whether osteoblasts discriminate cellular interactions with ECM ligands to mediate *opn* mRNA expression. Embryonic chicken calvaria osteoblasts were plated using identical cell numbers in plates coated with fibronectin (FN) (1mg/ml), collagen type I (Col1) (1mg/ml), denatured collagen type 1 (gelatin-G) (1mg/ml), osteopontin (OPN) (1mg/ml), vitronectin (VN) (1mg/ml), laminin (LN) (1mg/ml) or poly-L-lysine (pLp). Subsequently, a determination of the intracellular second signaling systems (protein kinases A and C) that are responsible for mediating the *opn* gene expression to cellular adhesion were determined. Finally, we evaluated he intracellular distrubution of focal adhesion kinase (FAK), p-tyrosine (PT) and vinculin (VC) on surfaces coated with FN or pLp. Results indicated that FN generated the strongest induction of OPN expression, followed by Col1 and LN. While G was weakly inducing, neither OPN, pLp or VN was capable of inducing *opn* mRNA expression, suggesting that the latter molecules did not facilitate specific cell adhesion. Induction of PKA and PKC occurred concomitantly only in osteoblasts from FN-coated dishes, while the other ligands promoted PKA solely. Thus, specific effects on kinase activities appear to be dependent on the selective ligand interactions, which are distinct form that of cell adhesion alone. Similarly, osteoblast adhesion did not appear to alter the general intracellular localization of FAC or VC. By contrast, a strong induction was noted for PT with generalized changes of its distribution throughout the cells after adhesion was promoted with a specific integrin binding ligand. Taken together with the *opn*-mediated gene expression results, these data suggest that induction of intracellular second signal kinase activity are related to the specific nature of the lignand's interaction with the receptor and les with the process of cellular-mediated adhesion alone.

Journal of Bone and Mineral Research
Vol 16, Suppl 1, September 2001, page S240
Abstract SA052

Appendix B

"The Predominant Integrin Ligands Expressed by Osteoblasts Show Preferential Regulation in Response to both Cell Adhesion and Mechanical Perturbation"
R.S. Carvalho, A. Bumann, J.L. Schaffer, L.C. Gerstenfeld

Accepted for publication in the Journal of Cellular Biochemistry, 2001

Final Report

DAMD17-98-1-8510

Bone-97 Mechanisms of Mechano-Transduction within Osteoblasts

P.I., Louis C. Gerstenfeld, Ph.D.

Journal of Cellular Biochemistry

September 4, 2001

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Dear Dr. Gerstenfeld:

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THE PREDOMINANT INTEGRIN LIGANDS EXPRESSED BY OSTEOBLASTS SHOW PREFERENTIAL REGULATION IN RESPONSE TO BOTH CELL ADHESION AND MECHANICAL PERTURBATION

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Summary: Previous studies demonstrated that both mechanical perturbation and cell adhesion induced the expression of osteopontin (*opn)* by osteoblasts [Carvalho et al., 1998]. The present study examined if these same stimuli on osteoblasts would induce the expression of other integrin binding proteins, specifically fibronectin (*fn*) and bone sialoprotein (*bsp*). All three genes showed 3 to 4 fold maximal induction in response to both cell adhesion and a single two hour period of an applied spatially uniform, dynamic biaxial strain of 1.3% at 0.25 Hz . Each gene, however, responded with a different time course of induction to mechanical strain, with *bsp*, *fn* and *opn* showing their maximal response at one, three and nine hours respectively after the perturbation period. In contrast, peak induction to cell adhesion was observed at 24 hours for *bsp* and *opn,* while *fn* levels peaked at 8 hours. Interestingly, while both *opn* and *fn* mRNA expression returned to base line after cell adhesion, *bsp* mRNA levels remained elevated. Examination of collagen type I and osteocalcin mRNAs showed unaltered levels of expression in response to either type of perturbation. A common feature of the signal transduction pathways, which mediate the gene expression in response to both cell adhesion and mechanical perturbation, was the activation of specific tyrosine kinases based on the ablation of the induction of these genes by the tyrosine kinase inhibitor genistein. While cycloheximide blocked the induction of all three mRNAs in response cell adhesion, it failed to block induction of any of these genes in response to mechanical perturbation. Such results suggest that the induction of these genes after mechanical perturbation was mediated by an immediate response to signal transduction, while cell adhesion mediated effects secondary to signal transduction. Depolymerization of microfilaments with cytochalasin D had no effect on the overall expression of any of these genes in response to cell adhesion and only blocked the induction of osteopontin expression in response to mechanical perturbation. These results suggest that cytoskeletal integrity is only selectively important in the signal transduction of certain types of stimuli and for the regulation of certain genes. In summary, both mechanical perturbation and cell adhesion

specifically stimulated the expression of integrin binding proteins. Furthermore, while there are common features in the signal transduction processes that mediate the induction of these genes in response to both stimuli, specific genes are separately regulated by specific mechanisms that are unique to both forms of stimuli.

INTRODUCTION

The mediation of cellular responses to mechanical stimuli depends in part on the recognition and interaction of selected cell-surface receptors with the extracellular matrix. Integrins are one class of cell surface receptors that mediate cell adhesion to the extracellular matrix. The integrins are a family of heterodimeric membrane receptors composed of multiple α and β isotypes. Variations in the extracellular domains of the individual isotypes of integrin receptors impart their specificity for specific extracellular matrix proteins [Hynes, 1992; Miyauchi, 1995]. The interaction of many integrin isotypes with their specific ligands is mediated by their recognition of the amino acid sequence arginine-glycine-aspartic acid-serine (RGDS) [Hynes, 1992]. It has been demonstrated that the binding of specific adhesion proteins to their integrin receptors generates a cascade of intracellular signals that are responsible for the regulation of a wide variety of cellular responses [Damsky and Werb, 1992]. Such interactions facilitate the appropriate functioning of essential cell functions such as cell adhesion, cell migration and survival of many cell types. These many functions are in turn mediated through integrin receptor activation of specific signal transduction mechanisms or by their mediation of structural alterations in the cytoskeletal architecture of cells [Juliano and Haskill, 1993].

In bone tissue, osteoblasts express high levels of several different RGD-containing proteins, the most predominant being osteopontin, bone sialoprotein and fibronectin [Gotoh et al., 1995; Puleo and Bizios, 1992; Winnard et al., 1995]. Osteopontin has been shown to interact with both osteoblasts and osteoclasts [Oldberg et al., 1988; Gotoh et al., 1990; Ross et al., 1993], and it is thought to play a role in mediating osteoclast resorption of bone tissue [Reinholt et al., 1990; Denhart and Guo, 1993]. The

expression of osteopontin is seen concurrently with alkaline phosphatase, and it has been identified as an early marker of osteoblast differentiation [Gerstenfeld et al., 1990]. Bone sialoprotein is another specific integrin ligand that is expressed by osteoblasts having a very restricted expression to only cells within the skeletal lineage and is seen predominantly in areas of mineralized growth cartilage and osteoid [Chen et al., 1994; Yang and Gerstenfeld, 1996; Yang and Gerstenfeld, 1997]. Bone sialoprotein has been shown to initiate calcification through its binding properties to collagen, calcium and hydroxyapatite [Hunter and Goldberg, 1994]. Unlike the former two proteins, fibronectin is expressed ubiquitously in most connective tissues. However, this protein appears to play an important role in the mechanisms of cell attachment, spreading and migration during early osteoblast differentiation [Winnard et al., 1995]. In addition to the RGDS peptide, fibronectin contains a synergistic adhesion site to the RGD sequence. It also contains two adhesion sites in the type III connecting segment of the whole molecule, the CS1 portion and the arginine-glutamic acid-aspartic acid-valine (REDV) sequence within the CS5 portion of fibronectin [Puleo and Bizios, 1992]. Some studies suggest that fibronectin also acts as an activator of cell adhesion rather than as a direct adhesion molecule [Curtis et al., 1992]. Thus fibronectin may mediate multiple interactions and responses by cells. Currently, there is a large body of data to suggest that the RGD-containing proteins osteopontin, bone sialoprotein and fibronectin play essential roles in the cellular differentiation and migration of osteoblasts during skeletal growth and/or in the initiation of spatial deposition of mineral in the extracellular matrix [Curtis et al., 1992; Hunter and Goldberg, 1993; Gerstenfeld et al., 1995; Schaffer et al., 1996].

Previous studies have shown that both the mechanical environment of osteoblasts and cell adhesion induce osteopontin gene expression [Toma et al., 1997; Carvalho et al., 1998]. These studies suggest that the interactions of cells with the extracellular matrix are integral components to the mediation of these stimuli. However, cellular perturbation through receptors may occur both through

the occupancy of the receptor as well as deformation through the engagement of the receptors with matrix attachment [Miyauchi et al., 1995]. Furthermore, it has been shown that specific reverse phosphorylation of specific proteins appears to regulate various intracellular pathways exclusively upon cellular attachment [Guan et al., 1991]. These results raise the possibility that signal transduction may be dependent on the matrix composition and that the cellular matrix components themselves may function as autocrine factors that regulate their own expression [Gerstenfeld, 1999]. Mechanical perturbation through receptor deformation and stimuli through receptor-ligation then may share overlapping molecular elements that mediate intracellular signal transduction and lead to common genomic responses. Such modulations may also involve cytoskeletal integrity and its relation with the integrin receptors. It has been hypothesized that cellular shape changes determine signal transduction pathways through the direct deformation of cellular membranes and reorientation of the microfilament network, thereby affecting integrin behavior [Ingber, 1991].

In previous studies from our laboratory, we have shown that that the continuous application of a dynamic, spatially uniform biaxial mechanical perturbation to osteoblasts leads to cytoskeletal rearrangement as well as changes in the extracellular matrix composition cellular response [Meazzini et al., 1998]. Other studies have shown that mechanical strain in cells of osteoblastic lineage only showed increased DNA synthesis when the cells were attached to specific ligands such as fibronectin [Wilson et al.,1995]. Thus, it seems that mechanical perturbation leads to both the physical alteration of ECM proteins and alterations in cellular architecture, which suggests that cells reach a homesostatic balance that is regulated through a complex set of receptor mediated interactions between the cells and the extracellular matrix proteins. In this study we examined if genes such as *opn, bsp* and *fn,* which specifically interact with integrins, would be commonly induced following either mechanical perturbation and/or cell-ligand binding. It was further examined if there are common signal transduction

pathways by which the expression of these proteins were modified following cellular attachment or mechanical perturbation, and if these pathways are unique or have overlapping mechanisms.

MATERIALS AND METHODS

Materials

All tissue culture supplies, cytochalasin D, colchicine, and cycloheximide were from Sigma Chemical Company (St.Louis, MO). H89 genistein was from LC Laboratories (Woburn, MA). Nylon membranes for Northern blots were from Biotrans, ICN Corp. (Aurora, OH).

Cell Culture

Seventeen-day embryonic chicken calvaria osteoblasts were and grown in culture as previously described [Gerstenfeld et al., 1988]. These cells were plated at a density of $2x10^6$ cells in 100 mm tissue culture dishes either left uncoated or coated with purified fibronectin (1mg/ml) as previously described [Schaffer et al., 1994]. Cultures were grown for two weeks until they reached confluence in minimum essential media supplemented with 10% fetal bovine serum (FBS). The medium was changed to BGJ_b supplemented with 10% FBS with the addition of 10mM β-glycerophosphate and 12.5 μg/ml ascorbic acid. All analyses were performed on at least three separate preparations of cells, and all data are presented as a percent increase in expression over that of the controls which were determined from parallel cultures grown under identical conditions. All error bars represent the standard deviation (SD) of the determinations from separate experiments and the number of replicates that were used for each measurement is denoted in each figure.

Mechanical Perturbation / Attachment Assays

The mechanical stretch apparatus used for these experiments was as previously described [Schaffer et al., 1994]. The design of the device imposes a verified temporal and spatial displacement profile to an optically transparent elastomeric membrane in which the strain magnitude was experimentally demonstrated to be homogeneous and isotropic (i.e. radial strain = circumferential strain

= constant over the culture surface) [Schaffer et al., 1994]. A polyurethane membrane (a generous gift of Dow Chemical Corporation, Midland, MI) was used in the culture dishes allowing for a constant 1.3% uniform biaxial strain at 0.25 Hz to be applied for a single two hour period. For each experiment, non-stimulated controls were performed on identical culture surfaces at the same time and from the same preparation of cells grown at identical conditions as the mechanically stimulated cultures. In all experiments for mechanical stretch, determinations were carried out six hours after the end of the two hour period of active cellular perturbation. For the attachment/integrin ligation assays, the cells were allowed to attach to fibronectin coated (1mg/ml) dishes, at the same concentration as those of mechanical stimulated cultures, for 24 hours. Fibronectin served as the basic ligand and uncoated plastic plates served as controls.

Signal Transduction Studies

Signal transduction pathways that mediate the cell responses of the mechanical perturbation and/or attachment/ligation were investigated by the use of specific chemical inhibitors. The final concentration for each of these compounds was: 50 mM cycloheximide, 20 mg/ml genistein, 1 μM H89 (Sigma), 50 μM cytochalasin D (Sigma) and 1 μM colchicine (Sigma). Cycloheximide and genistein were incubated for 30 minutes, while cytochalasin D was incubated for 1 hour and colchicine for 6 hours. Controls were separately determined for each compound in cultures treated identically with the various compounds but in which the cells were either not mechanically stimulated or were attached to uncoated dishes.

Isolation and Analysis of RNA

Total RNA was isolated using tri-Reagent™ (Molecular Center, Cincinati, OH) according to the manufacturer's instructions. RNA was resolved on 1% agarose gels containing 2.2 M formaldehyde [Toma et al., 1997] and 5 mg of total RNA was loaded per gel/lane. Chicken cDNA's used for these studies were pro α1[I] collagen [Lehrach et al., 1979], osteocalcin [Nuegelbaurer et al., 1995],

osteopontin [Moore et al., 1991] and bone sialoprotein [Yang et al., 1995]. Northern blots with ^{32}P cDNA-labelled probes were carried out at 65°C in 2.5xSSC, 50 mM Na-phosphate buffer, 100 μg/ml single stranded salmon sperm DNA, and for 18 to 24 hours in a rotating hybridization oven (Robins Scientific, Sunnyvale, CA). Autoradiograms were quantified using an LKB Ultra II scanning densitometer (LKB, Broma, Sweden) and values were normalized against 18 S ribosomal RNA obtained by hybridization of each blot to a conserved nucleotide sequence probe of 18 S ribosomal subunit (Ambion Corp., Austin, TX). All analyses were performed at least three times, and all data are presented as a percentage in expression over that of the control, which were determined from parallel cultures. All data were evaluated as a mean ± 2 standard deviations with a minimum of three experiments from different populations of primary cells, and appropriate statistical analysis were performed.

RESULTS

Osteoblast-adhesion and mechanical perturbation increase levels of RGD-containing proteins:

Initial studies were carried out to assess the expression of mRNA levels for osteopontin, bone sialoprotein and fibronectin osteoblasts following either cell adhesion or mechanical perturbation. Fibronectin was used as the adhesion substrate in these experiments. The temporal profiles of *opn* expression were shown to peak at 24 hours. This induction was 3 to 4 fold above that of control samples. At time periods beyond 24 hours, there was a sharp reduction in *opn* expression, which returned to its baseline levels of expression (Figure 1). Bone sialoprotein expression followed a similar profile to that seen for *opn* also peaking at 24 hours (3 fold). However, there was no marked decrease in the expression of *bsp*, which remained elevated (2 fold) at 48 and even 72 hours (Figure 2). In contrast, the expression of *fn* mRNA started at significantly higher levels when compared to the other two mRNAs (Figure 3). Expression of *fn* peaked at 8 hours from the onset of the perturbation (2 fold), and showed a sharp reduction to its baseline levels soon thereafter.

The expression of these mRNAs was then examined after the application of mechanical perturbation. As expected, mechanical perturbation of osteoblasts increased *opn* expression by 2 to 3 folds, peaking at 9 hours post-stretch (Figure 1). This clearly contrasted with adhesion, which showed a peak induction in *opn* expression at 24 hours. In the case of *fn,* mechanical perturbation also showed an increase in expression peaking at 3 and 6 hours from the onset of the perturbation (Figure 3). It is interesting to note, however, that mechanical perturbation was inhibitory for *bsp* expression. In Figure 2, *bsp* levels started at 2 fold of control samples at 1 hour post-stretch and quickly decreased to baseline levels at 9 hours post-stretch.

These results would suggest that integrin binding-ECM molecules are selectively responsive to perturbation via either mechanical perturbation or cell adhesion mediated signal transduction. Two other prevalent ECM genes, collagen type I and osteocalcin, were then examined as a comparison to these RGD containing integrin ligands. Both collagen type I (*col1*) and osteoclacin (*oc*) mRNAs were examined after mechanical perturbation. Interestingly, neither one of these genes showed alterations in their expression when the cells were subjected to mechanical perturbation (Figure 4).

Different matrix proteins require different signal transduction pathways following cell adhesion:

In order to further understand if a common signal transduction processes mediated the induction of the three RGD integrin ligand genes, pharmacological inhibitors of specific signal transduction pathways were used. From the slow increase in *opn* levels following cell adhesion, it appears that this protein is involved in a secondary, down-stream event to other genomic changes. Indeed, the compound cycloheximide, a known inhibitor of *de novo* protein synthesis, blocked the induction of *opn* mRNA expression following adhesion to fibronectin (Figure 5). Cycloheximide also inhibited the expression levels of *fn* and *bsp* genes (Figure 6 and 7), showing that these genes were also dependent on new protein synthesis following cellular adhesion. Even though the maximum levels of *fn* occurred at 9

hours post cellular adhesion, this finding was consistent with the relative long period for maximum induction of *opn* and *bsp* (Figures 1, 2 and 3).

Previous observations had shown that changes in *opn* mRNA expression in response to mechanical perturbation were dependent on the integrity of the microfilament structure of the cell [Toma et al., 1997]. This finding is consistent to the results seen in this study (Figure 5). The role of the cytoskeleton in the signal transduction pathways for each of these mRNAs was examined in these studies. Incubation of the osteoblast cultures with the microtubule depolymerizing agent colchicine did not affect the expression of any of the mRNAs (Figures 5, 6 and 7). However, cultures treated with cytochalsin-D, a microfilament disruption agent, inhibited the levels of *opn* mRNA below those of control levels (Figure 5), following mechanical perturbation. This change was not seen for either *bsp* or *fn* mRNAs (Figures 6 and 7).

Finally, specific inhibitors for second messenger systems were used. The use of genistein, a potent inhibitor of tyrosine kinase phosphorylation, was shown to significantly inhibit the expression of *opn, bsp* and *fn* mRNAs. As shown in Figure 5, this finding has also been observed previously for *opn* expression in mechanically stimulated cells [Toma et al., 1997; Carvalho et al., 1997]. Genistein treatment in cells subjected to mechanical perturbation also inhibited the levels of *fn* and *bsp* mRNAs (Figures 6 and 7). The pharmacological inhibitor of PKA-like kinases, H-89, also caused an inhibition of *fn* levels, but it did not effect the expression of *opn* or *bsp* following cell adhesion. However, when H-89 was given to cells that had undergone mechanical perturbation, the expression of both *bsp* and *opn* mRNAs were inhibited, but not that of *fn* mRNA (Figures 5, 6 and 7). These findings suggest that the perturbation in expression of the RGD-containing extracellular proteins, which are studied here in response to cell adhesion, is distinct from that of mechanical perturbation, yet both responses appear to be uniquely dependent on the activation of specific subsets of kinases.

DISCUSSION

In previous studies we have shown that both mechanical perturbation and cell adhesion led to the induction of increased *opn* expression. While both types of stimuli were dependent on integrin receptors, each was mediated by a specific set of intracellular signals that were distinct for each type of perturbation [Carvalho et al., 1998]. The present study provides further evidence that both cellular adhesion and mechanical perturbation lead to the selective induction of multiple integrin binding proteins within osteoblasts. Furthermore, while these data suggest that there may be some common mechanisms of signal transduction that stimulate the increased expression of these genes, each of the genes was uniquely and separately regulated by both stimuli (Table 1).

In the bone extracellular matrix, RGD-containing glycoproteins including osteopontin, bone sialoprotein and fibronectin are presumed to interact with cell adhesion receptors (integrins) on the surface of the bone cells [Grzesik and Robey, 1994]. Studies suggest that that these extracellular components directly affect gene expression [Pienta et al., 1991], which takes place following mechanical perturbation [Resnick et al., 1993; Toma et al., 1997; Carvalho et al., 1998]. When considering adhesion separately from mechanical perturbation, one needs to take into account the effects of the former over the latter, as it may be speculated then that adhesion or integrin-ligation acts as a "primer" prior to any response due to the mechanical perturbation. In particular, it is interesting to note that the effects of the dynamic, spatially uniform mechanical perturbation on cells that are in the process of adhesion enhances the formation of new receptor-ligand bonds. In studies by Kuo et al. [1993], mechanical perturbation was shown to alter the kinetic regulation of cell adhesion, thus interfering with binding rate. However, once the cells are adherent, specific RGD tri-peptides inhibited both adhesion and mechanical perturbation responses [Carvalho et al., 1998]. Wilson et al. [1995] also presented evidence that RGD peptides, fibronectin and certain integrin antibodies disrupted integrin-ligand

interaction, which in turn ablated strain induced mechano-transduction responses, without disrupting adhesion of the same cells.

It has been proposed that receptors such as integrins can behave as a homeostatic system for modulating the extracellular matrix structure and organization in response to the structural needs of the cell [Werb et al., 1989; Gerstenfeld, 1999]. In this context, it is interesting to note that the mechanical forces that are applied to tissues should be the structurally deformed at their anchorage points to the extracellular matrix (ECM). These sites should be the focal points at which mechanical strains are specifically transmitted to the cells. The strains to which a cell is subjected, therefore, will be effected by both the compositional properties of the matrix and the receptors on the surfaces of the cells, which can interact with these ECM proteins. The importance of extracellular interactions for the process of mechano-sensation has been shown by Du et al. [1996]. In those experiments, it was demonstrated that isolated genes of the touch receptor neurons in C. elegans encoded for extracellular proteins. These authors [Du et al., 1996] further hypothesized that the extracellular matrix mediates the anchoring properties of specialized cells, enabling their mechano-sensory response.

Concerning the intercellular components of mechano-signal transduction, both Davies et al. [1993] and Ingber [1997] have suggested that the transduction of mechanical stimuli in anchorage-dependent cells is due to a combination of signal transduction processes via the cytoskeleton through integrin receptors that interact with the cytoskeleton, as well as through biochemical signals. In order for mechano-signal transduction to occur through the cytoskeleton, it is necessary that cooperative interactions occur between the three component parts of cytoskeleton; the micofilaments, intermediate filaments and microtubles. Studies of osteoblasts have also shown that the cytoskeleton changes both its structural architecture and its composition in response to mechanical perturbation [Meazzini et al., 1998]. However, signal transduction of different types of signals may not depend on the integrity of all of these structural elements. In this study, this is shown by following the selective inhibition of

microtubules and microfilaments. The results presented in Figure 5 and Table I demonstrate that *opn*–cell adhesion dependent mRNA expression was inhibited following colchicine treatment, yet adhesion was not affected after the addition of cytochalasin-D. The inverse effect was seen when the cells were mechanically stimulated [Toma et al., 1997]. In contrast, alterations in cytoskeletal architecture via pharmacological manipulation did not change the expression of *bsp* or *fn* mRNAs in response to either mechanical perturbation or cell adhesion. These latter results suggest the involvement of microfilaments in the selective regulation of some genes but not others. Indeed, recent studies have shown that the induction of COX 2 enzyme expression within osteoblasts in response to fluid flow mediated shear stress was also regulated via alteration in the cells cytoskeletal architecture [Pavalko et al., 1998]. Such results suggest that there may be specific subsets of genes that are commonly regulated and are dependent on the cytoarchitecture of the cell, yet others may not be dependant on the cytoarchitecture, even though they are regulated by cell adhesion or mechanical perturbation.

This differentiation between mechanical perturbation and adhesion appears to follow unique mechanisms. Since each extracellular matrix ligand interacts with different integrins, it may be speculated that selective interactions or mediation of the various signals occurs through specific integrin receptors. For instance, while $\alpha_V\beta 3$ deficient cell populations were not capable of migrating in response to *opn*, these same cells did migrate significantly in response to fibronectin and vitronectin [Liaw et al., 1995]. Furthermore, specific ligand interactions may mediate a variety of intracellular signals ranging from ion flux to selective G-protein kinase and/or phosphatase activation. The transduction of the mechanosignal at the cellular membrane leads to a cascade of downstream signaling events, many of which are mediated by tyrosine kinases, which in turn phosphorylate other kinases [Berk et al., 1995]. Kinases that have been associated with mechano-transduction include mitogen-activated protein kinase (MAPK). MAPK has been shown to follow mechanical perturbation in cardiac cells [Yamazaki et al., 1993] and fluid flow in endothelial cells [Tseng and Berk, 1993]. It has been suggested that such

responses are part of a multiplicity of pathways and might be grouped functionally into those that are either calcium dependent or calcium independent [Berk et al., 1995; Ishida et al., 1997]. The presence of calcium is important for the activation of a putative shear stress receptor (membrane level), which regulates a pertussis toxin-sensitive G protein-coupled K^+ channel (SSR) [Ohno et al., 1993] and the enzyme phospholipase C [Nollert et al., 1990]. The levels of PIP_2, in turn will be regulated by rho, a small GTP-binding protein [Chong et al., 1994]. The calcium-independent pathway involves the activation of MAPK [Berk et al., 1995]; however, other calcium-independent tyrosine kinases such as src and FAK may also be involved in the shear stress transduction.

A common feature in the signal transduction processes that regulate the expression of all the integrin binding genes in response to either adhesion or mechanical perturbation was the inhibition of the induction of their expression by genistein. This suggests that a tyrosine kinase(s) is involved in the signal transduction, which stimulates the expression of all of these proteins. In contrast, PKA inhibition through H-89 treatment demonstrated a selective effect for *opn* and *bsp* in response to mechanical perturbation but not with cell adhesion (Figures 5 and 6). As for *fn*, H-89 inhibited the effects of adhesion but not those of mechanical perturbation (Table 1; Figure 7). In the experiments reported herein, there were not any changes in the non-RGD containing proteins collagen type I and osteocalcin (Figure 4). Integrin receptors have been described as potential mediators of mechanical perturbation [Ingber et al., 1994; Ishida et al., 1997]. Activation of integrins has been shown to induce the tyrosine phosphorylation of FAK at focal adhesion complexes [Schaller et al., 1994]. In addition, other proteins within these focal adhesion contacts, such as paxillin and src, will also be phosphorylated when exposed to flow [Girard and Nehem, 1993; Bull et al., 1994]. Our laboratory has shown that FAK phosphorylation was regulated by mechanical perturbation [Toma et al., 1997], also suggesting that the disruption of microtubules does not affect the expression of any gene studied following mechanical perturbation. This is an interesting finding as it relates to MAPK, as this kinase has been shown as the

earliest signal activated by flow at physiological stress [Tseng and Berk, 1993]. MAPK is also known as a microtubule-associated kinase [Sabe et al., 1994], suggesting a role in the cytoskeleton. However, as disruption of microtubules did not affect gene expression following perturbation, one may only speculate on the role of MAPK as a mechanical perturbation-dependent kinase. On the other hand, we have observed that adhesion alone in the presence of the microtubule-disrupting drug colchicine, blocked the induction of *opn* expression in particular. Thus, it is conceivable that MAPK plays a role in this mechanism, since this kinase has been shown to be activated by cell binding to fibronectin [Morino et al., 1995]. It has been suggested that activation of integrins is associated with the same signal events that occur when cells are exposed to flow [Vuori and Ruoslahti, 1993; Schwartz and Deninghoff, 1994; Beck et al., 1995]. However, further study is needed, as the complexity of such a response can not be understood if the responses of mechanical perturbation and adhesion are not taken into account individually.

Integrin-ligation is thought to stimulate the same signal events as mechanical perturbation [Berk et al., 1995]. Indeed, we have shown here that this is the case, even though the mechanisms that mediate both responses are uniquely different. If integrins are the mediators for mechano-transduction in both forms of activation, then there maybe several different integrin receptors acting in concert with other sensors that are specific to the mechanical activation. The dependency of mechanical perturbation effects on RGD-containing proteins in this study and the lack of response in either collagen type I or osteocalcin further demonstrate an active role of integrins in adhesion and perturbation. It is clear that integrins and focal contacts play important roles in mechano-transduction. It remains to be determined, however, how the mechanisms of adhesion cross talk with those of mechanical perturbation and which kinases and second signals are common in regulating downstream events prior to any activation in gene expression.

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Table I. Comparison of signal transduction pathways for the mRNA expression of *opn*, *bsp* and *fn* between cell adhesion and mechanical stretching.

Genes	*opn*		*bsp*		*fn*	
Perturbation	**Adhesion**	**Stretch**	**Adhesion**	**Stretch**	**Adhesion**	**Stretch**
Time after peak expression	24 hrs	9 hrs	24 hrs	1 hr	8 hrs	3 hrs
De novo protein synthesis	yes	no	yes	no	yes	no
Tyrosine kinase-mediated	yes	yes	yes	yes	yes	yes
PKA-mediated	no	yes	no	yes	yes	no
Requires microfilaments	no	yes	no	no	no	no
Requires microtubules	yes	no	no	no	no	no

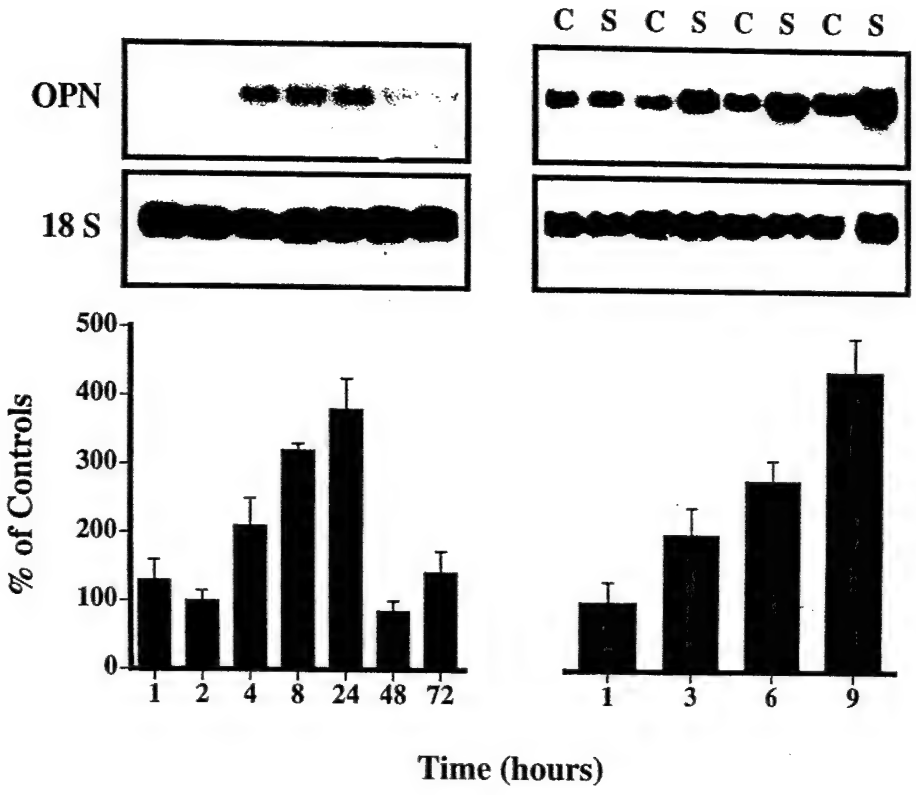

Figure 1. Effect of cell adhesion and mechanical perturbation on the temporal expression of *opn* mRNA expression by osteoblasts. Northern blot analysis of osteopontin mRNA expression following cell adhesion of osteoblasts to fibronectin is seen in the left and that of induction in response to mechanical perturbation is seen on the right. The expression of the 18S rRNA is seen in the lower panel of each figure. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 2, 4, 8, 24, 48 and 72 hours after an initial 24 hour period of attachment relative to the control samples at identical times. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 3, 6 and 9 hours post mechanical perturbation. Controls=(C) and strained samples =(S). All data are presented as percent induction of expression of the mRNAs relative to their control samples. Error bars are the S.D. determined from at least three experiments.

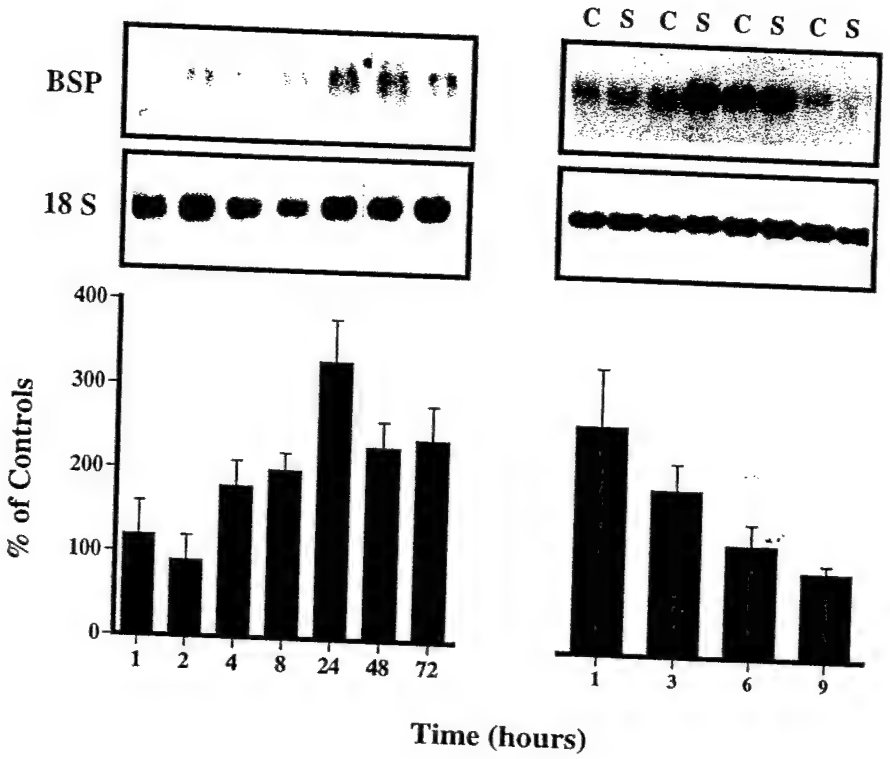

Figure 2. Effect of cell adhesion and mechanical perturbation on the temporal expression of *bsp* mRNA expression by osteoblasts. Northern blot analysis of bone sialoprotein mRNA expression following cell adhesion of osteoblasts to fibronectin is seen in the left and that of induction in response to mechanical perturbation is seen on the right. The expression of the 18S rRNA is seen in the lower panel of each figure. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 2, 4, 8, 24, 48 and 72 hours after an initial 24 hour period of attachment relative to the control samples at identical times. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 3, 6 and 9 hours post mechanical perturbation. Controls=(C) and strained samples =(S). All data are presented as percent induction of expression of the mRNAs relative to their control samples. Error bars are the S.D. determined from at least three experiments.

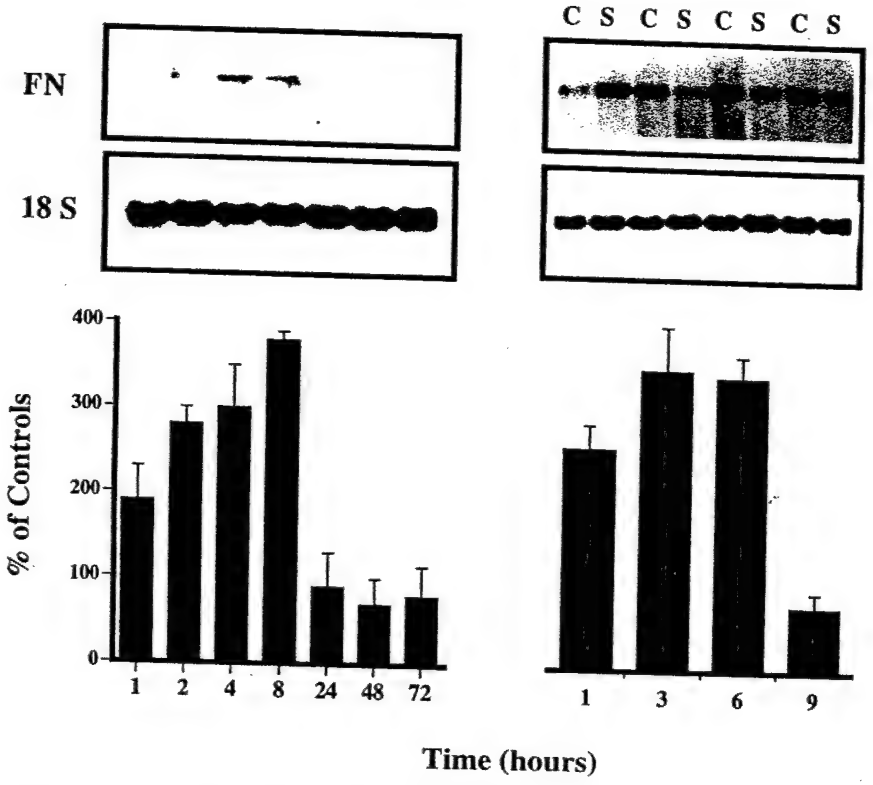

Figure 3 Effect of cell adhesion and mechanical perturbation on the temporal expression of *fn* mRNA expression by osteoblasts. Northern blot analysis of fibronectin mRNA expression following cell adhesion of osteoblasts to fibronectin is seen in the left and that of induction in response to mechanical perturbation is seen on the right. The expression of the 18S rRNA is seen in the lower panel of each figure. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 2, 4, 8, 24, 48 and 72 hours after an initial 24 hour period of attachment relative to the control samples at identical times. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 3, 6 and 9 hours post mechanical perturbation. Controls=(C) and strained samples =(S). All data are presented as percent induction of expression of the mRNAs relative to their control samples. Error bars are the S.D. determined from at least three experiments.

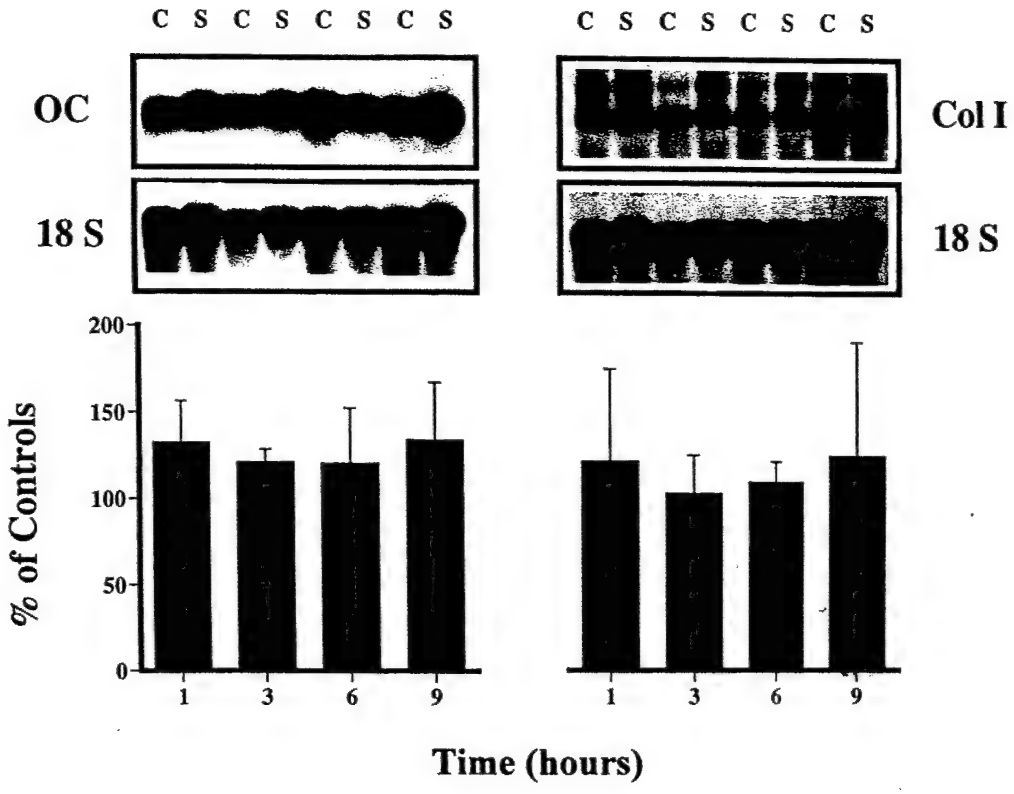

Figure 4. Effect of mechanical perturbation on the temporal expression of *col1* and *oc* mRNA expression by osteoblasts. Northern blot analysis of osteocalcin and collagen type I mRNA expression following mechanical perturbation of osteoblasts are denoted in the figure. The graphic representation of the temporal expression for these mRNAs is shown by the times of 1, 3, 6 and 9 hours post perturbation. All the panels show the percent induction of expression of the steady mRNA levels relative to their control samples. Controls=(C) and strained samples =(S). Error bars are the S.D. determined from at least three experiments.

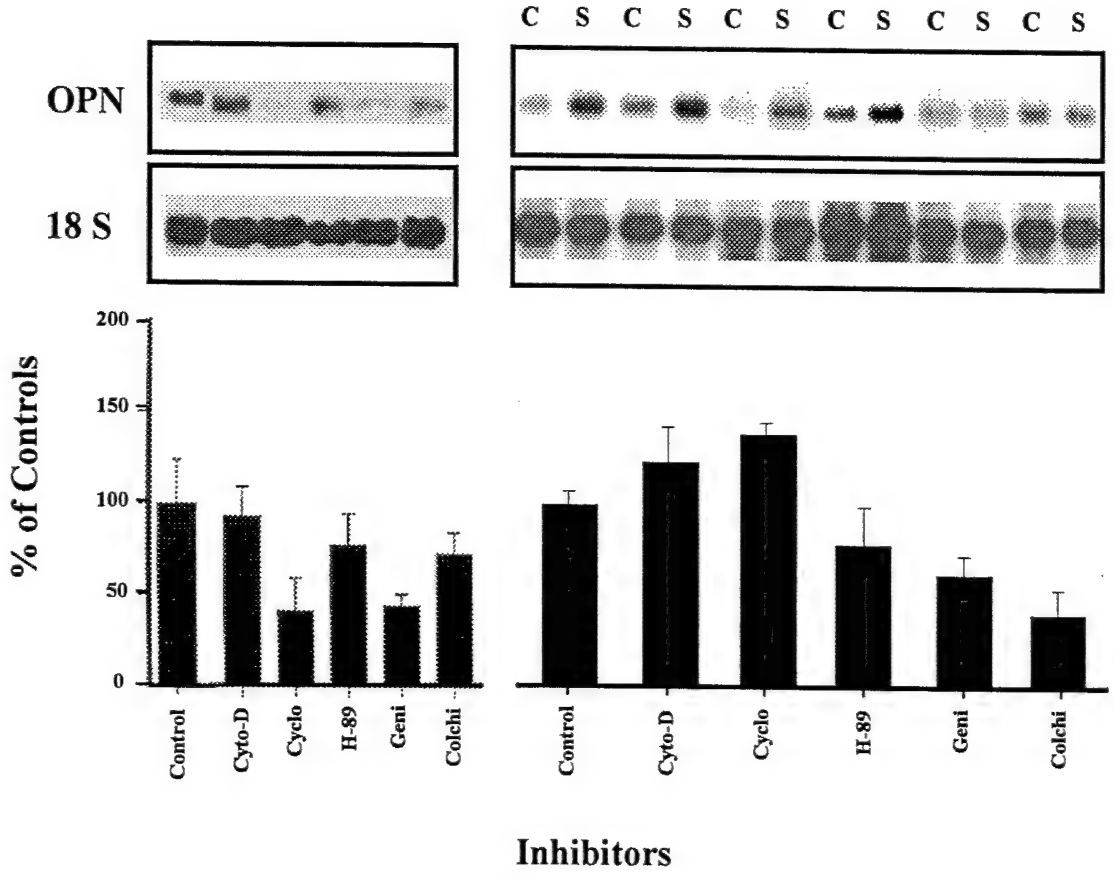

Figure 5. Effect of pharmacological inhibitors on the mRNA expression of *opn* by osteoblasts in response to cell adhesion and mechanical perturbation. Effects of various second messenger inhibitors and cytoskeletal disrupters on the expression of *opn* mRNA in response to cell adhesion and mechanical perturbation were examined. Cells were treated with the microfilament depolimerizing agent cytochalasin-D (Cyto-D), the microtubule disrupting agent colchicine (Colchi), the protein synthesis inhibitor cycloheximide (Cyclo), the PKA inhibitor H-89 (H-89) and the tyrosine kinase inhibitor genistein (Geni). Autoradiographs for the Northern blot analysis of the steady state levels of each mRNA and of the 18 S RNA are presented separately. Graphic analysis shows the percent induction or inhibition of the various mRNAs compared to that of controls. Error bars are the S.D. of three experiments.

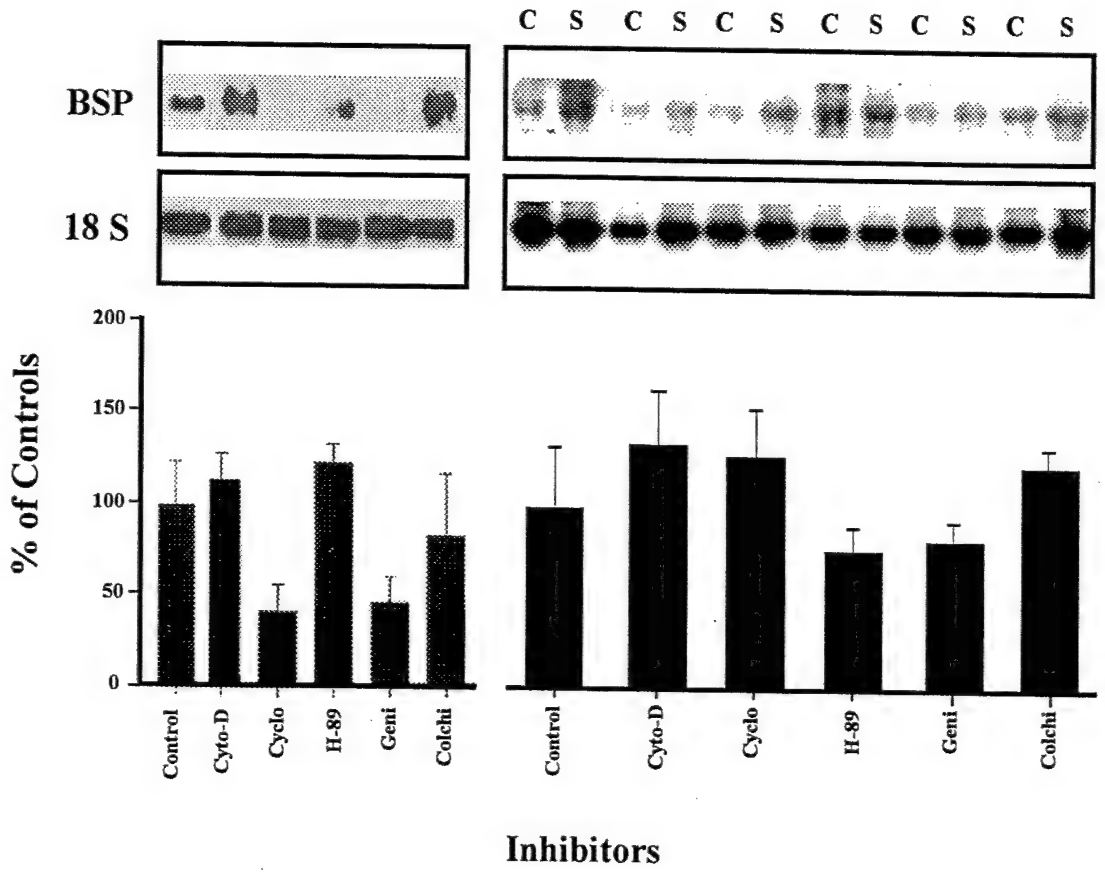

Figure 6. Effect of pharmacological inhibitors on the mRNA expression of *bsp* by osteoblasts in response to cell adhesion and mechanical perturbation. Effects of various second messenger inhibitors and cytoskeletal disrupters on the expression of *bsp* mRNA in response to cell adhesion and mechanical perturbation were examined. Cells were treated with the microfilament depolimerizing agent cytochalasin-D (Cyto-D), the microtubule disrupting agent colchicine (Colchi), the protein synthesis inhibitor cycloheximide (Cyclo), the PKA inhibitor H-89 (H-89) and the tyrosine kinase inhibitor genistein (Geni). Autoradiographs for the Northern blot analysis of the steady state levels of each mRNA and of the 18 S RNA are presented separately. Graphic analysis shows the percent induction or inhibition of the various mRNAs compared to that of controls. Error bars are the S.D. of three experiments.

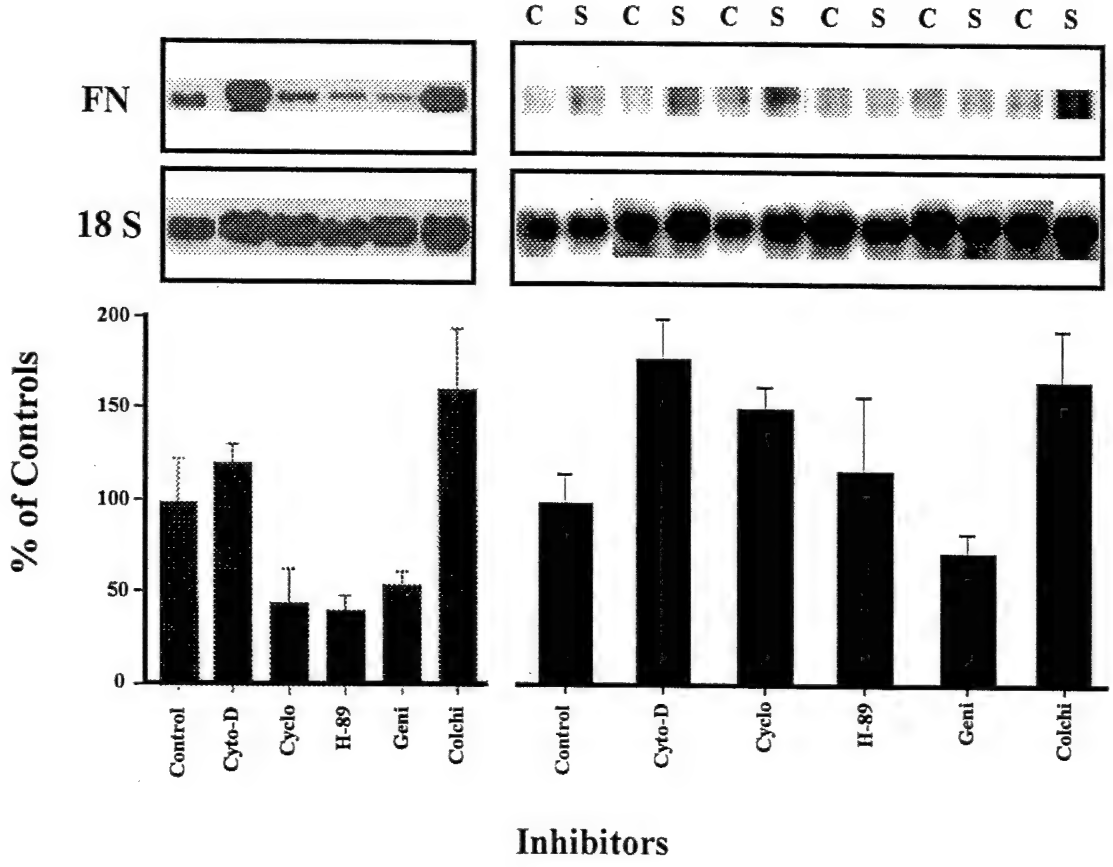

Figure 7. Effect of pharmacological inhibitors on the mRNA expression of *fn* by osteoblasts in response to cell adhesion and mechanical perturbation. Effects of various second messenger inhibitors and cytoskeletal disrupters on the expression of *fn* mRNA in response to cell adhesion and mechanical perturbation were examined. Cells were treated with the microfilament depolimerizing agent cytochalasin-D (Cyto-D), the microtubule disrupting agent colchicine (Colchi), the protein synthesis inhibitor cycloheximide (Cyclo), the PKA inhibitor H-89 (H-89) and the tyrosine kinase inhibitor genistein (Geni). Autoradiographs for the Northern blot analysis of the steady state levels of each mRNA and of the 18 S RNA are presented separately. Graphic analysis shows the percent induction or inhibition of the various mRNAs compared to that of controls. Error bars are the S.D. of three experiments.

Appendix C

"Differential Induction of Osteopontin Gene Expression by Osteoblasts in Response to Adhesion to Selective Integrin Ligands"
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Bone-97 Mechanisms of Mechano-Transduction within Osteoblasts

P.I., Louis C. Gerstenfeld, Ph.D.

DIFFERENTIAL INDUCTION OF OSTEOPONTIN GENE EXPRESSION BY OSTEOBLASTS IN RESPONSE TO ADHESION TO SELECTIVE INTEGRIN LIGANDS

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Running title: RGD-proteins, integrins and adhesion

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ABSTRACT: Bone formation and remodeling are partly regulated by the mechanical environment of the skeletal cells. One of the mechanisms by which mechanical stimuli may be mediated, is through cellular deformation that is transmitted through focal contact points of cell adhesion with the extracellular matrix. Previous studies have shown that osteopontin (*opn*) expression is commonly stimulated in response to both cellular adhesion. The current studies examined whether osteopontin mRNA expression in response to adhesion was mediated by specific integrin ligands. Embryonic chicken calvaria osteoblasts were plated using identical cell numbers on culture surfaces coated with fibronectin (FN) (1mg/ml), collagen type I (Col1) (1mg/ml), denatured collagen type 1 (gelatin-G) (1mg/ml), osteopontin (OPN) (1mg/ml), vitronectin (VN) (1mg/ml), laminin (LN) (1mg/ml) or poly-L-lysine (pLp) or albumin (AL). Selective adhesion was mediated by FN, COL1, G, and LN but not by pLP, AL, VN or OPN. FN generated the strongest induction of OPN expression, followed by Col1 and LN. While G was weakly inducing and neither OPN, VN, pLp or AL was capable of inducing *opn* mRNA expression. A determination of the intracellular second signaling systems (protein kinases A and C) that were activated in response to adhesion showed that both PKA and PKC activities were increased concomitantly in osteoblasts after adhesion to FN, while the other ligands that promoted cell adhesion only increased PKA activity to varying degrees. Thus, specific effects on kinase activities appear to be dependent on the selective ligand interactions, which were distinct from that of cell adhesion alone. A final set of studies evaluated the intracellular distribution of several protein components known to be within focal adhesion complexes, (FAK), and vinculin (VC) and assayed the distribution of p-Tyrosine (PT) containing proteins found within cells that were aloowed to adhere either to surfaces coated either with FN or pLp. Adhesion led to the strong induction of PT with generalized change of PT containing protein

from the cell surface to the nucleus. In contrast while VC and FAK while showing increased intensity did not show any change in surface distribution. Taken together with the *opn*-mediated gene expression results, these data suggest that induction of intracellular second signal kinase activity are related to the specific nature of the lignand's interaction with the receptor and less with the process of cellular-mediated adhesion alone. They also show that the predominat integrin type that promoted both osteoblast ahesion and osteropontin gene induction was are β1 integrin and not β3 integrin binding ligands.

INTRODUCTION

The bone remodeling cycle is known to be intimately involved in the metabolic homeostasis of mineral balance (Jilka and Manologas, 1994). Bone formation and the remodeling cycle have been shown to be essential in maintaining the structural integrity of skeletal tissue in response to the mechanical loading to which it is subjected (Lanyon et al., 1982; Lanyon and Rubin, 1987). Bone remodeling has also been hypothesized to provide the means of repairing bone tissue that has been damaged as a result of mechanical fatigue (Mori and Burr, 1993). Thus, it may be speculated that skeletal cells which mediate the remodeling process are regulated by their mechanical environment. It has been shown that most cell types are subjected to various forms of loading, including shear stresses due to fluid flow, stresses due to pressure, and other mechanical stimuli such as tensile stresses exerted by neighboring cells, the extracellular matrix (ECM) and the internal tension of the cytoskeleton (Shyy and Chien, 1997). Thus, in order for osteoblasts to respond to their mechanical environment, they must in some way interpret the signal generated by the stimuli. One mechanism by which cells sense these signals is through the physical deformation of the tissue (Carvalho et al., 1998). Currently, many reports have shown that signal transduction of biochemical signals following stimulation involves cell surface ligands, such as integrins (Ingber, 1991; Wang et al., 1993; Carvalho et al., 1998; Urbich et al., 2000). This is evidenced by the fact that mechanical stimulation-derived signals can only be perceived by osteoblasts and other responsive cells after cell adhesion (i.e. integrin-mediated) has taken place. Moreover, some of the signal transduction and gene expression events activated by mechanical stresses are similar, if not identical, to those induced by integrin-mediated cell adhesion (Shyy and Chien, 1997). This may be explained by changes in cell shape caused by either form of stimulation resulting in the

reorientation of the microfilament network affecting integrin behavior (Ingber, 1991). However, receptor-ligation stimulations also appear to be dependent on the positional parameters provided by the matrix. Physical alteration of ECM proteins may also change integrin conformation, facilitating the activation of specific signal transduction molecules which in turn regulate cytoskeletal arrangement and cellular response (Meazzini et al., 1997). Integrin ligands may in fact, have an autocrine and/or a paracrine function in regulating cellular function in the ECM (Gerstenfeld, 1999) and ECM proteins themselves may function as initiators of cellular activation (Carvalho et al., unpublished observations).

In bone tissue, osteopontin (OPN) is one of the predominant RGD-containing ECM proteins (Gotoh et al., 1995) and it is ubiquitously expressed in all skeletal tissues during embryogenesis (Gerstenfeld; 1999). Ultrastructural localization of OPN in areas adjacent to resorptive osteoclasts (Reinholt et al., 1990), suggests that this protein has an important role in anchoring osteoclasts to bone allowing for the resorption process. In addition, osteoclasts have been shown to synthesize OPN during active bone remodeling (Dodds et al., 1995) which further demonstrates that OPN has a major role in the maintenance of bone homeostasis (Gerstenfeld et al., 1999). We have previously shown that both mechanical stimulation (Toma et al., 1997; Carvalho et al., 1998) and cellular adhesion (Carvalho et al., 1998) result in *opn* induction, and that integrin receptors may have a common role in the signal transduction processes of both forms of stimulation. Thus, we wanted to know how osteoblasts would discriminate "ligation" at a molecular level by using different ECM "ligands" to study cell-mediated adhesion.

In order to investigate this question, it is important to understand the potential effect of selected ECM proteins in response to processes of cellular adhesion alone. Consequently, in

this study we have tested a number of ligands through attachment assays of chicken calvarial osteoblasts on tissue culture surfaces coated with fibronectin (FN), native collagen type I (Col1), denatured collagen type I (i.e. gelatin (G)), osteopontin (OPN), vitronectin (VN), laminin (LN) or polyL-Lysine (PL). Subsequently, a determination of the intracellular second signaling systems (Kinases) that are responsible for mediating cellular adhesion were determined. The selective induction of PKA and PKC occurred concomitantly only in osteoblasts from FN-coated dishes, while the other ligands promoted PKA solely. These results show that specific effects on kinase activities are dependent on the selective ligand interactions, which are distinct than those of cell adhesion alone. In addition, the strong inhibitory effect of G-coated dishes on kinase activity as measured here, indicates that biological processes that are mediated by these ligands are related to their structural conformation with their interaction with specific receptors. We also evaluated the intracellular distribution of focal adhesion kinase (FAK), phosphotyrosine (PT) and vinculin (VN) on surfaces coated with FN or PL. These results suggest that osteoblast adhesion on either surface did not appear to alter the general intracellular localization of FAC or VN. By contrast, a strong induction was noted for PT with a generalization change of its distribution throughout the cells after adhesion was promoted with a specific integrin binding ligand.

Finally, we examined if integrin interactions with the same specific ligands described above were involved in the process of *opn* expression following cellular adhesion. These experiments showed that FN generated the strongest induction of *opn* expression followed by Col1 and LN. While G was weakly inducing, neither OPN nor VN was capable of inducing *opn* mRNA expression, suggesting that the latter did not facilitate specific cell adhesion. We hypothesize that other ECM proteins in addition to OPN, that are ligands for these receptors

are themselves regulated in response to cellular interaction with the matrix. Taken together with the *opn*-mediated gene expression results, these data suggest that induction of intracellular second signal kinase activity are related to the specific nature of the ligand's interactions with the receptor and less with the process of cellular-mediated adhesion alone.

MATERIALS AND METHODS

Materials

All tissue culture supplies, were from Sigma Chemical Company, St.Louis, MO. Antibodies mouse anti-chicken Vinculin, rabbit anti-human focal adhesion kinase, $p125^{FAK}$, mouse pTyr, goat anti-mouse FITC conjugated and sheep anti-rabbit Cy3 conjugated were also from Sigma Chemical Company, St.Louis, MO. Nylon membranes for Northern blots were from Biotrans, ICN Corp. Aurora, OH.

Cell Culture

Seventeen-day embryonic chicken calvaria osteoblasts were grown in culture as previously described (Gerstenfeld et al., 1988). These cells were plated at a density of 2×10^6 cells in 100 mm tissue culture dishes as previously described (Schaffer et al., 1994). Cultures were grown for two weeks until they reached confluence in minimum essential media supplemented with 10% fetal bovine serum (FBS). The medium was changed to BGJb supplemented with 10% FBS with the addition of 10mM β-glycerophosphate and 12.5 μg/ml ascorbic acid. All analyses were performed on at least three separate preparations of cells and all data is presented as a percent increase in expression over that of the controls which were determined from parallel cultures grown under identical conditions. All error bars represent the standard deviation (SD) of the determinations from separate experiments and the number of replicates that were used for each measurement is denoted in each figure.

Attachment Assays

For the adhesion assays the cells were initially grown one week in DME media and then subcultivated onto petri dishes that were coated with 2 $\mu g/cm^2$ or 10 $\mu g/cm^2$ of each of the proteins studied here. Fibronectin (FN), vitronectin (VN), laminin (LN), denatured collagen (i.e. gelatin) (G) and collagen type I (Col1) were obtained from Sigma Chemical Company, St Louis, MO. Osteopontin (OPN) was purified as described by Gotoh et al. (1995). FN served as the basic ligand. Dishes coated with polyL-lysine (PL) or bovine serum albumin (Al) (also from Sigma) served as controls. Different proteins were added to tissue culture plates under sterile conditions and incubated for 24 hours at 37°C. Cells were allowed to attach for one, four or twenty four hours and assays were performed at least three times for each protein ligand. Values were calculated as percentage of the cells on the experimental surfaces relative to the cell numbers observed on the plates coated with PL.

Signal Transduction Studies

Signal transduction pathways that mediate cell responses as a result of attachment/ligation were investigated measuring the specific kinase activity of the cells plated into each separate protein coating. General protein kinase C (PKC) and protein kinase A (PKA) activities were measured using specific fluorescent substrates. Enzyme activities were first normalized per total protein content used in each assay. Values were then calculated as the percentage of activities seen in cells plated on polyL-lysine coated dishes. Controls were separately determined for each compound, in cultures treated identically with the various compounds but in which the cells were attached to uncoated dishes.

Immunohistochemical studies

The intracellular distribution of focal adhesion kinase (FAK), phosphotyrosine (PT) and vinculin (VN) were examined after adhesion on surfaces coated with either PL or FN. Cell layers were washed with phosphate buffer saline (PBS) and fixed in 3.7% paraformaldehyde for 5 minutes at 4°C. Cells were then permeabilized with 0.02% triton X-100 in PBS for 10 minutes and background was blocked with blocking solution (Pierce). Cells were washed in washing buffer (0.05% casein acid hydrolysate, 0.015% Tween 20) for 10 minutes and incubated with primary antibodies (mouse anti-chicken Vinculin, rabbit anti-human FAK or mouse PT antibody for 45 minutes at 37°C. Once again cells were washed with washing buffer, incubated with secondary goat anti-mouse FITC conjugated antibodies or sheep anti-rabbit Cy3 conjugated antibodies for 30 minutes at room temperature, washed and visualized/photographed under the light microscope.

Isolation and Analysis of RNA

Total RNA was isolated using tri-ReagentTM (Molecular Center, Cincinati, OH) according to the manufactures instructions. RNA was resolved on 1% agarose gels containing 2.2 M formaldehyde (Toma et al., 1997) and 5 mg of total RNA was loaded per gel/lane. Chicken cDNA used for these studies was that of osteopontin (Moore et al., 1991). Northern blots with P^{32} cDNA-labelled probes were carried out at 65°C in 2.5 X SSC, 50 mM Na phosphate buffer, made 100 µg/ml single stranded salmon sperm DNA, and for 18 to 24 hours in a rotating hybridization oven (Robins Scientific, Sunnyvalle, CA). Autoradiograms were quantified using an LKB Ultra II scanning densitometer (LKB, Broma, Sweden) and values were normalized to the 18 S ribosomal RNA obtained by hybridization of each blot to a conserved nucleotide sequence probe of 18 S ribosomal subunit (Ambion Corp., Austin, TX).

All analyses were performed at least three times and all data is presented as a percentage in expression over that of the controls determined from parallel cultures. All data were evaluated as the mean ± 2 standard deviations with a minimum of three experiments from different populations of primary cells and appropriate statistical analysis were performed.

RESULTS

Osteoblasts have variable adhesion properties to different ligands: Initial studies were carried out to determine if chicken calvaria-osteoblasts demonstrate selective properties to different extracellular matrix proteins that serve as cell ligands. Osteoblasts were allowed to attach for four hours to FN, C, G, LN, OPN, VN or Al (used as a control). These results are seen in Figure 1, panels A and B. These data show that the different ECM proteins, FN, C and G promoted selective adhesion, while three other proteins VN, LN and OPN did not. As can be seen from these data, FN was the most effective protein in promoting selective adhesion. In addition, both natured and denatured collagen also promoted adhesion, however, these showed saturation at between 1 and 3 $\mu g/cm^2$ of surface coating. In contrast, FN did not show saturation in promoting cell adhesion until about 30 $\mu g/cm^2$ (data not shown). These results demonstrate that adhesion of osteoblasts is specifically promoted by different types of integrin ligands. Due to the response of FN in promoting osteoblast adhesion, this protein was selected as the basic cell ligand for the immunohistochemical studies described below.

Selective induction of kinase activity is related to specific protein ligand: The relationship of cell adhesion to the selective induction of specific intracellular kinase activities was examined next. In this study, cell adhesion was carried out at a fixed concentration of coating (10 $\mu g/cm^2$), considered the best concentration in preliminary observation (data not shown).

Figure 2, Panels A and B demonstrate that the profiles of selectively mediated attachment of embryonic osteoblasts was seen after only 60 minutes, compared to the profiles in Figure 1 in which attachment had been assessed for 4 hours. The induction of two classes of kinases, protein kinase A (PKA) and protein kinase C (PKC), that are involved in second signaling were then determined in identically prepared parallel cultures. It is interesting to note that attachment to FN showed induction of both PKA and PKC activities, while Col1 showed the strongest induction of PKA activity with no induction of the activity of PKC. In addition, OPN also stimulated PKA activity contrasting to G and LN, which strongly inhibited PKA activity. These results show that specific effects on second signaling kinase activities are dependent on the specific ligand interactions and not on the processes of cell adhesion (Figures 1 and 2). Indeed, the strongest inhibitory effect of G on both PKA and PKC activities while at the same time promoting cell adhesion suggests that the biological processes that are mediated by these proteins are related to the structural conformation of these ligands and their interactions with specific receptors.

Fibronectin stimulates the distribution of phosphotyrosine following osteoblast adhesion: Since FN was shown to be the most effective attachment ligand, this protein was selected to mediate cell adhesion on the distribution of three structural and enzymatic components found with focal adhesion complexes. Figure 3 shows the intracellular distribution of focal adhesion kinase (FAK), phosphotyrosine (PT) and vinculin (VN) on surfaces coated with FN or polyL-lysine (PL). These data showed that adhesion to surfaces coated with either molecule did not appear to alter the general intracellular localization or distribution of FAK nor VN within an one-hour period. On the other hand, there was a strong induction of PT levels and a generalization of its distribution throughout the cells after adhesion was promoted with a

specific binding ligand as seen in FN coating. These data reinforce the conclusion reached at Figure 2 that suggests that the induction of intracellular kinase activities are related to the specific nature of the ligand's interactions with its receptor and less with the process of cellular adhesion alone.

Attachment of osteoblasts to different ECM proteins selectively increase the expression of *opn*: In this study we examined if cell adhesion to various integrin ligands would regulate the expression of the bone-specific protein OPN. These studies also examined if integrin interactions with specific ligands were involved in these processes. The results are depicted in Figure 4. As can be seen in this figure, induction of *opn* mRNA was seen as early as 4 hours after the cells had been allowed to adhere and continued to increase up to 24 hours. At 4 hours of cell attachment, only coatings of FN and C resulted in an increase in *opn* mRNA expression. At the second time point of 24 hours, it is interesting to note that once again coating with C, FN and to a lesser extent LN generated an increase in *opn* mRNA expression. These induction were significant with a 4 fold increase following osteoblast attachment to FN and almost 3 fold increase following osteoblast attachment to C. By contrast, results showed that G, OPN and VN did not increase *opn* mRNA, therefore, these did not appear to facilitate specific cell adhesion. Taken together, these results suggest that receptor binding to different ECM proteins constitutes cellular attachment and that these selectively induce expression of *opn*.

DISCUSSION

The experiments presented here define how osteoblasts discriminate ECM-mediated signals at a molecular level. In this study we have investigated whether specific cell surface receptors that interact with ECM matrix proteins (i.e. native collagen, osteopontin, fibronectin,

denatured collagen, vitronectin and laminin) use common mechanisms in the regulation of the cellular response to adhesion.

ECM is an important structural scaffold for the organization of living tissues in the body, thus providing an array of information for several key cellular functions, such as cell motility, polarity, migration, proliferation (Damsky and Werb, 1992; Schwartz et al., 1995; Damsky, 1999) and survival (Frisch and Francis, 1994; Damsky, 1999; Urbich et al., 2000), thus preventing apoptosis (Zhang et al., 1995; Globus et al., 1998). These functions require specialized ECM proteins for a number of cell types including osteoblasts. The resultant process of osteoblast-ECM adhesion generates an abundance of signals designed to modulate vital osteoblastic responses. Thansduction of signals from the ECM takes place through a variety of transmembrane proteins, including the integrin family of cell receptors (Clark and Brugge, 1995; Schwartz et al., 1995 for a review). Integrins are heterodimers comprised of two different subunits called α and β, each one of which numbering over 20 different types (Ingber, 1991). Consequently, the specificity of integrin binding will be dependent on the unique combination of these subunits (i.e. integrin $\alpha_5\beta_1$ binds fibronectin, while $\alpha_2\beta_1$binds collagen) (Ingber, 1997).

In order to investigate cell-ECM adhesion, a number of integrin-binding proteins were selected. For instance, we selected OPN and FN since these have been shown to interact with integrins in the cell surface of bone, and are RGD-containing glycoproteins (Grzesik and Robey, 1994). In addition to the FN integrin receptor $\alpha_5\beta_1$, VN was also chosen as a ligand due the central role of its receptor ($\alpha_v\beta_3$) in cell migration and the support of cell survival (Aplin et al., 1999; Urbih et al., 2000). Similarly, LN was selected since this protein was also shown to prevent ECM-dependent apoptosis in mature osteoblasts (Globus et al., 1997).

The results presented here demonstrate that FN, C and G promoted osteoblast adhesion, while three other proteins VN, LN and OPN did not, with FN being the most effective protein in promoting selective adhesion (Figure 1). This is consistent with previous studies that also showed preferential adhesion of osteoblasts to FN and low affinity to LN (Gronthos et al., 1997). Overall, adhesion of osteoblasts appeared to be transduced primarily through integrin receptors. While, these results indicate that osteoblast adhesion is specifically promoted by β_1 and not by β_3 containing integrins, these are suggestive of a further discrimination between the individual integrin ligands since LN did not strongly mediate cell adhesion. By contrast, studies of osteoblast-like cells adhering to biomimetic peptide surfaces during shorter time periods (i.e. 30 minutes) than the ones described here (i.e. one hour >) show that in addition to β_1, β_3 integrin may also govern cell attachment to RGD-coated surfaces (Rezania and Healy, 1999). It is not known whether shorter time points would yield similar results in our model, however, longer time points (i.e. 4 hours) in our study clearly indicated that β_1 was the primary integrin ligand (Figure 1). Figure 1 also shows that coating of C and G promoted adhesion, however, these showed saturation at between 1 and 3 $\mu g/cm^2$ of surface coating, whereas dishes coated with FN did not result in saturation by promoting cell adhesion until about 30 $\mu g/cm^2$ (data not shown). The latter may reflect affinity of the integrin-peptide interaction changes due to the microenvironment of the ligand as suggested by Houseman and Mrksich (2001). These authors also suggested that weaker interactions display a density-dependent enhancement of binding during cell attachment and spreading. Interestingly, when studying the cell adhesive shear strength and cell detachment surface energy, Yamamoto et al. (2000) reported that collagen and fibronectin-coated surfaces elicited the highest values respectively, when compared with non-specific bonding materials such as glass and polystyrene. While

these observations are in agreement with our results, these authors concluded that the number of binding sites is more important that the strength of each binding.

ECM protein-receptor specificity is an integral part of cellular signal transduction providing the means for initiation of second messenger signaling cascades (Schaller and Parsons, 1994, Damsky, 1999) which in turn go on to regulate cellular function. As a result, we subsequently looked at key signaling mechanisms following osteoblast-ECM protein adhesion. Based on the relationship of the ligand-mediated adhesion with the second signal kinase activity, Figure 2A shows that kinase activities are dependent on the specific ligand interactions and not on the processes of cell attachment alone. This is evidenced by the inhibitory effect of G coating on both PKA and PKC pathways, while at the same time promoting cell adhesion. Thus, the processes of cellular adhesion most likely are mediated solely through the RGD or other small integrin binding motifs, but the down stream activation of intracellular signaling responses are specifically related to the structural conformations of the intact ligand. In Figure 2B, one can appreciate that the activity of PKA fluctuated much more than that of PKC (i.e. 2-3 fold increase in C as opposed to no changes in LN-coated dishes). While OPN did not promote cell adhesion, it did stimulate PKA activity, while G and LN both strongly inhibited PKA activity. By contrast, the activity of PKC did not appear to change very much with exception of FN-coating, which showed a 2-fold increase versus that of control levels. These results on the second signaling kinase activities prompted us to further investigate the signaling pathways following basic osteoblast adhesion and FN was chosen as a basic ligand. The latter also stems from the fact tat FN appeared to have elicited the best response in terms of cell attachment (Figure 1). In addition, it has been shown that osteoblasts

strongly depend on FN for survival once these start to form bone nodules (Globus et al., 1998), suggesting that FN may be a regulator of apopotosis in osteoblasts.

The response of osteoblast adhesion in its dependency to the ECM substrate onto which the cell adheres seen here has similar aspects to cell responses such as those elicited by mechanical stimulation (Wilson et al., 1995; Carvalho et al., 1998). Our laboratory and others have demonstrated that physical deformation of attachment substrata markedly affects protein distribution, which may or may not be transient (Meazinni et al., 1998 for a review). It is also known that ECM-derived signals are transduced to the cells through transmembrane molecules (i.e. integrins) primarily in zones of focal adhesions, which are known areas in which signal transduction takes place (Clark and Brugge, 1995; Burridge and Chrzanowska-Wodnicka, 1996). Therefore, signaling cascades were further investigated by the immunolabeling of two well-known focal adhesion molecules, focal adhesion kinase (FAK) and vinculin (VN) and of phosphotyrosine (PT). Although, FAK is a protein that is commonly associated with zones of focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996, Kano et al., 1999), osteoblast adhesion to FN did not appear to change its distribution (Figure 3). This result is contrasting evidence between the distinction of signal transduction of cellular adhesion to those of derived from mechanical stimulation. In the latter, FAK molecules localize to plaque-like areas of a $\alpha_v\beta_3$ integrins (Wosniak et al., 2000). It has also been shown that FAK autophosphorylation may be directly related to cell binding to OPN (Liu et al., 1997), which is consistent with the role of OPN in anchoring functional osteoclasts during bone remodeling (Reiholt et al., 1990). The role of FAK appears, however, to be highly relevant in ECM-mediated signal transduction. It has been shown that FAK transduces survival signals from ECM and that these are monitored by p53 (Ilic et al., 1998). It may be that lack of FAK redistribution following

adhesion reflects the early stage of the embryonic osteoblast phenotype studied here. In addition, PKC may also be activated by the interruption of survival signals from the ECM in conjunction with p53 (Damsky, 1999). The latter infer a close relationship between FN attachment and osteoblast survival, which is consistent with our results that showed the highest PKC activity response for dishes coated with FN as opposed to all the other ligands (Figure 2B). Since protein tyrosine phosphorylation occurs very often during intracellular signaling, the levels of phosphotyrosine were examined. Anti-phosphotyrosine staining was detected throughout the cells (Figure 3), however, it appeared mostly concentrated over areas of focal adhesions (not shown) suggesting more active events in these areas. Immediately following cell adhesion to FN, there was a strong induction of PT levels and a generalization of its distribution throughout the cells. It is speculated that this response may be directly related to β_1 integrin-mediated binding, such as the one seen when osteoblasts were subjected to mechanical stress in vitro (Bierbaum and Notbohm, 1998).

Finally, we investigated the molecular activation of intracellular signaling by looking at the osteoblast-ECM adhesion-mediated signals through their regulatory expression of *opn*, the predominant RGD-containing protein in bone (Gotoh et al., 1995). It is know that *opn* expression is regulated by both adhesion and mechanical stimulation (Toma et al., 1997; Carvalho et al., 1998). In these studies, the induction of *opn* expression at 24 hours after the cells were plated on tissue culture plastic alone or coated with these various extracellular matrix molecules. The strongest induction was seen with FN, C and LN while denatured collagen was weakly inducing and neither G, OPN nor VN was capable of inducing *opn* mRNA expression (Figure 4). These results suggest that the same integrin receptors that facilitate specific cell attachment also facilitate the induction of *opn* expression. It is

interesting to note that neither OPN nor VN mediate either of these cellular responses suggesting that the latter are not facilitated through a $\alpha_v\beta_3$ receptor, corroborating the adhesion assay results shown in Figure 1. The one difference that was observed, however, was in the comparison of cell attachment on G vs. the induction of *opn* expression. This result may suggest that signal transduction through the collagen receptor is only mediated when it interacts with native collagen. Other ligands not studied here may also offer clues as to the cell-matrix interaction following adhesion-activated signals. Byzova et al. (2000) showed that activation of $\alpha_v\beta_3$ receptor enhanced adhesive and migratory responses of human endothelial vein cells to bone sialoprotein, an important RGD-containing glycoprotein of bone cells (Grzesik and Robey, 1994). It has been suggested that certain ECM ligands (i.e. osteopontin) seem to act as autocrine factors that modify not only cell behavior in response to changes in matrix composition (Gerstenfeld, 1999) but also those changes seen following mechanical deformation (Carvalho et al., unpublished observations) by having a structural role in the matrix itself. The maintenance of the compositional balance of these various proteins in the ECM appears to maintain tissue homeostasis in response to its structural needs. In other words, the spatial and temporal assembly of ECM ligands constitutes the informational content of these molecules and characterizes the induction of cellular response following adhesion and potentially other key biological processes.

Throughout this paper, we have speculated that integrins are the mediators of ECM-osteoblast attachment, even though we have not looked directly at integrin receptors or their expression. However, the conclusion that integrins are transducing signals from the ECM is consistent with many observations published in the literature (Clark and Brugge, 1995; Gronthos et al., 1997; Liu et al., 1997; Carvalho et al., 1998; Damsky, 1999; Yamada and

Geiger, 2000; Zimmerman et al., 2000). Specifically, we have stated that β_1 was the primary integrin ligand (Figure 1). This conclusion is supported by recent experiments that showed transgenic cells expressing a dominant-negative truncated integrin β_1 subunit driven by an osteocalcin promoter in order to target its expression to mature osteoblasts and osteocytes (Zimmerman et al., 2000). In the latter studies, while wild type cells promoted osteoblast differentiation by high levels of osteocalcin and alkaline phosphatase activity, the majority of transgenic cells lost attachment and died, which further suggests that β_1 integrin is very important in the regulation of cellular adhesiveness. It is important to point out, however, that our study deals only with a small number of signaling pathways in view of the number of those describing ECM-induced integrin-dependent signaling that have been previously reported. Some of these include mitogen-activated protein kinase activation, Ca^{++} influx, pH alterations and inositol phosphate turnover (Yamada and Geiger, 1997). The investigation of these may lead to additional clues as to the regulatory influences of ECM attachment proteins.

In conclusion, adhesion of osteoblasts to ECM proteins appears to be specifically mediated by the β_1 class of integrins, in particular that of adhesion to FN through $\alpha_5\beta_1$. This same class of integrins appears to be responsible for the signal transduction process that stimulates *opn* induction. The β_3 integrin containing-ligands vitronectin and osteopontin neither mediate specific adhesion nor induce *opn* gene expression. Therefore, even though cellular attachment may occur through non-specific binding, down stream activation of intracellular signaling responses that are mediated by ECM ligands are specifically related to the ligand's structural conformation and its interactions with specific receptors.

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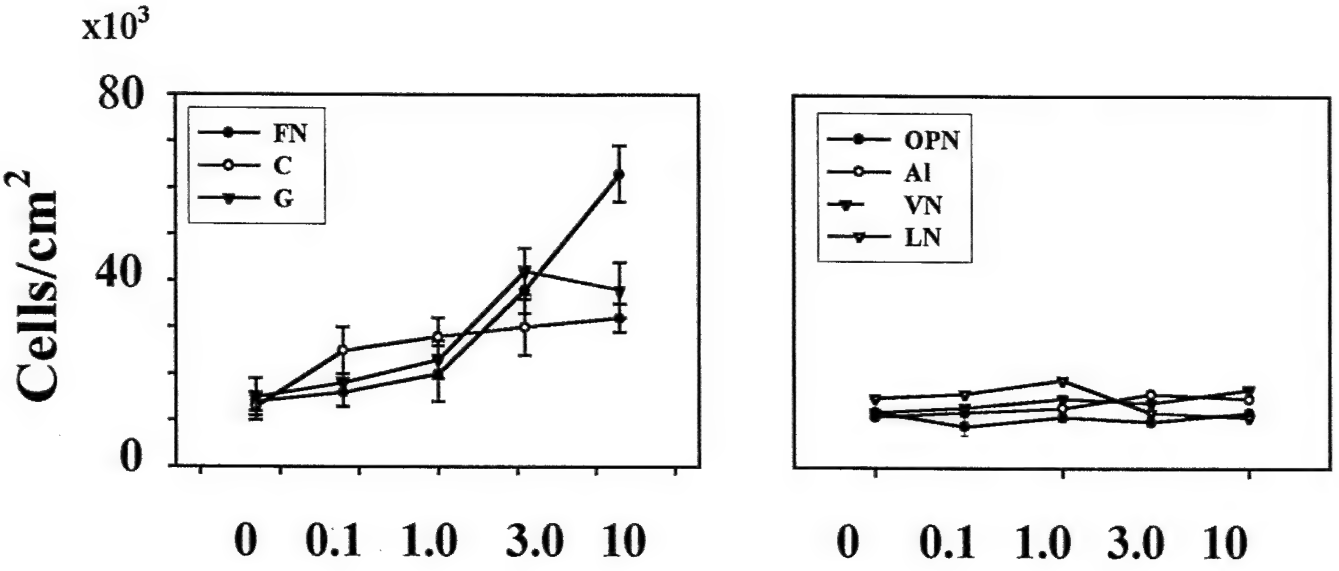


Figure 1. Relationship of ligand concentration to cell attachment. Bacterial petri dishes were coated with 0, 0.1, 1.0, 30 and 10 μg/cm^2 of each of the proteins. G=gelatin, denatured collagen, VN=vironectin, FN=fibronectin, OP=osteopontin, Al=bovine serum albumin, C=fibrillar collagen, native collagen, PL=polyL-lysine. Attachment was for 4 hours. Values were calculated as the total number of cells attached per cm^2 of coated surface area.

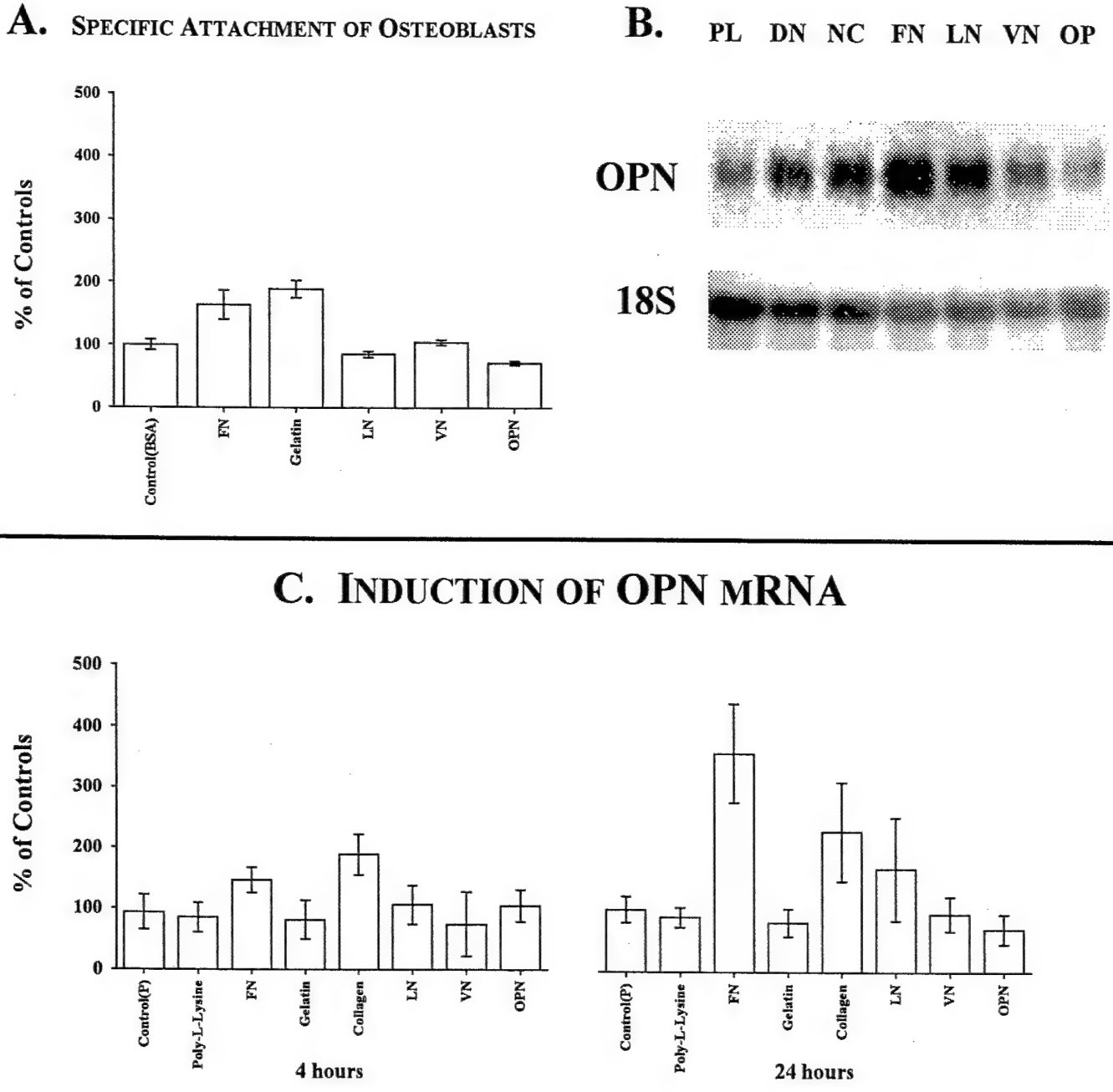


Figure 2. Comparison of selective attachment with induction of osteopontin in response to cell plating on different integrin ligands. Panel A Selective attachment of 17 day embryonic chick osteoblasts on different ligands after a four hour period. Left panel depicts the percent attachment compared to control on poly l-lysine coated surfaces after hours. All protein concentrations were at $2\mu g/cm^2$. Right panel depicts the total number of cells that were attached after four hours as a function of varying the concentration of the ligands.

Panel B. Induction of osteopontin expression in response to cell attachment on various ECM proteins. Osteopontin expression was measured by Northern blot analysis and the band intensities were normalized to the 18s band(data not shown). Left panel shows percent induction after four hours and right panel after 24 hours. The nature of the protein coatings are denoted in the figure

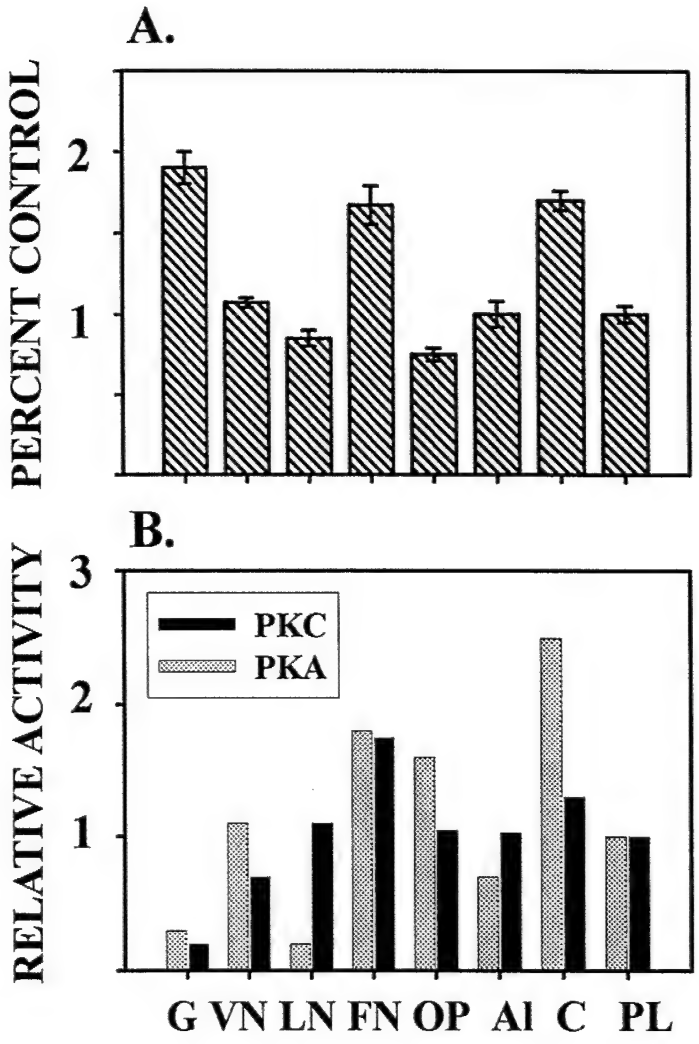
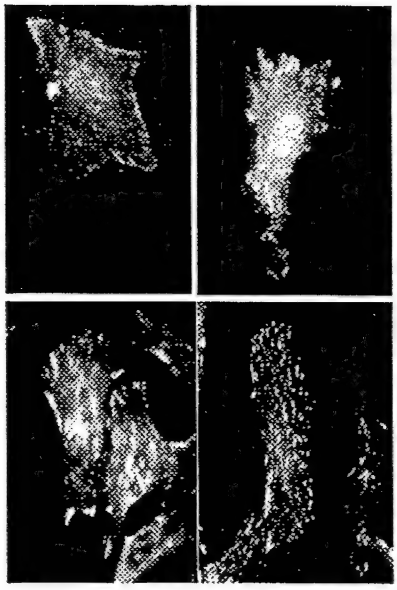


Figure 3. Relationship of Ligand Mediated Adhesion and Second Signal Kinase Activity. **Panel A**. Percent osteoblast attachment on selective protein coating. Petri dishes were coated with 2 $\mu g/cm^2$ of each of the proteins. G=gelatin, denatured collagen, VN=vironectin, FN=fibronectin, OP=osteopontin, Al=albumin, C=fibrillar collagen, native collagen, PL=polyL-lysine. Attachment was for 1 hours. Values were calculated as the percentage of cells on the experimental surfaces relative to the cell numbers observed on the polyL-lysine coated dishes.

Panel B Kinase activity after adhesion on selective protein coatings. General PKA and PKC activities were measured using specific fluorescent substrates. Enzyme activities were first normalized per total protein content used in each assay. Values were then calculated as the percentage of activities seen in the cells plated on the polyL-lysine coated dishes.



Appendix D

"Induction of a Neoarthrosis by Precisely Controlled Motion in an Experimental Mid-Femoral Defect"

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Induction of a neoarthrosis by precisely controlled motion in an experimental mid-femoral defect

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Abstract

Bone regeneration during fracture healing has been demonstrated repeatedly, yet the regeneration of articular cartilage and joints has not yet been achieved. It has been recognized however that the mechanical environment during fracture healing can be correlated to the contributions of either the endochondral or intramembranous processes of bone formation, and to resultant tissue architecture. Using this information, the goal of this study was to test the hypothesis that induced motion can directly regulate osteogenic and chondrogenic tissue formation in a rat mid-femoral bone defect and thereby influence the anatomical result. Sixteen male Sprague Dawley rats (400 ± 20 g) underwent production of a mid-diaphyseal, non-critical sized 3.0 mm segmental femoral defect with rigid external fixation using a custom designed four pin fixator. One group of eight animals represented the controls and underwent surgery and constant rigid fixation. In the treatment group the custom external fixator was used to introduce daily interfragmentary bending strain in the eight treatment animals (12° angular excursion), with a hypothetical symmetrical bending load centered within the gap. The eight animals in the treatment group received motion at 1.0 Hz, for 10 min a day, with a 3 days on, one day off loading protocol for the first two weeks, and 2 days on, one day off for the remaining three weeks. Data collection included histological and immunohistological identification of tissue types, and mean collagen fiber angles and angular conformity between individual fibers in superficial, intermediate, and deep zones within the cartilage. These parameters were compared between the treatment group, rat knee articular cartilage, and the control group as a structural outcome assessment. After 35 days the control animals demonstrated varying degrees of osseous union of the defect with some animals showing partial union. In every individual within the mechanical treatment group the defect completely failed to unite. Bony arcades developed in the experimental group, capping the termini of the bone segments on both sides of the defect in four out of six animals completing the study. These new structures were typically covered with cartilage, as identified by specific histological staining for Type II collagen and proteoglycans. The distribution of collagen within analogous superficial, intermediate, and deep zones of the newly formed cartilage tissue demonstrated preferred fiber angles consistent with those seen in articular cartilage. Although not resulting in complete joint development, these *neoarthroses* show that the induced motion selectively controlled the formation of cartilage and bone during fracture repair, and that it can be specifically directed. They further demonstrate that the spatial organization of molecular components within the newly formed tissue, at both microanatomical and gross levels, are influenced by their local mechanical environment, confirming previous theoretical models.

Introduction

Joint diseases such as osteoarthritis or traumatic arthritis are characterized by the loss of articular cartilage and altered joint anatomy with resulting pain, incapacitation, and substantially lowered quality of life. While total joint arthroplasty is a successful treatment for these conditions, it is not a lasting solution for the young active person. Indeed, the repair of articular cartilage would be a major advance in the treatment of arthritic diseases. However, although bone regeneration during fracture healing has been demonstrated repeatedly, the regeneration of a joint structure and its articular cartilage remain elusive goals [7,8,12,24].

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It has long been recognized though that the mechanical environment influences the formation of cartilage, bone, and resultant tissue architecture during skeletal healing and in response to normal activity. The mechanical environment has been implicated in the upregulation of cartilage matrix synthesis both experimentally [16,30] and as a result of exercise [28]. Moreover, the specific development of cartilage versus bone in healing fractures is related to the mechanical environment, a concept supported by theoretical models [2,4,6,9,11,18,27].

Fracture repair involves the interaction of cells with their local mechanical environment in a cascade of molecular events, resulting in a repaired and remodeled fracture site [6,14,29]. A comparable series of interactive events also occurs in limb joint ontogeny [3,5,21,22]. Indeed, it has been demonstrated that the normal in vivo development of a joint depends on a relatively consistent mechanical load being applied [3,13,18–22]. Models of non-union have even been developed using manually applied mechanical stimulation [1]. Yet, despite the general understanding that the mechanical environment plays an important role in tissue development and repair, incorporation of mechanical stimulation into studies of cartilage and bone repair have been limited.

This study was undertaken as an empirical test of the hypothesis that precise mechanical stimulation, induced on a daily basis, can regulate osteogenic and chondrogenic tissue formation in a rat femoral defect model. Our study was designed as a confirmation of theoretical models of mechanobiological influences during skeletal healing. We demonstrate that the differentiation processes of cartilage and bone during fracture healing can be experimentally manipulated into desired tissue and structural level outcomes. This study may provide the basis for future research approaches to examine the molecular regulatory processes by which the mechanical environment regulates cartilage and bone formation, and potentially joint formation.

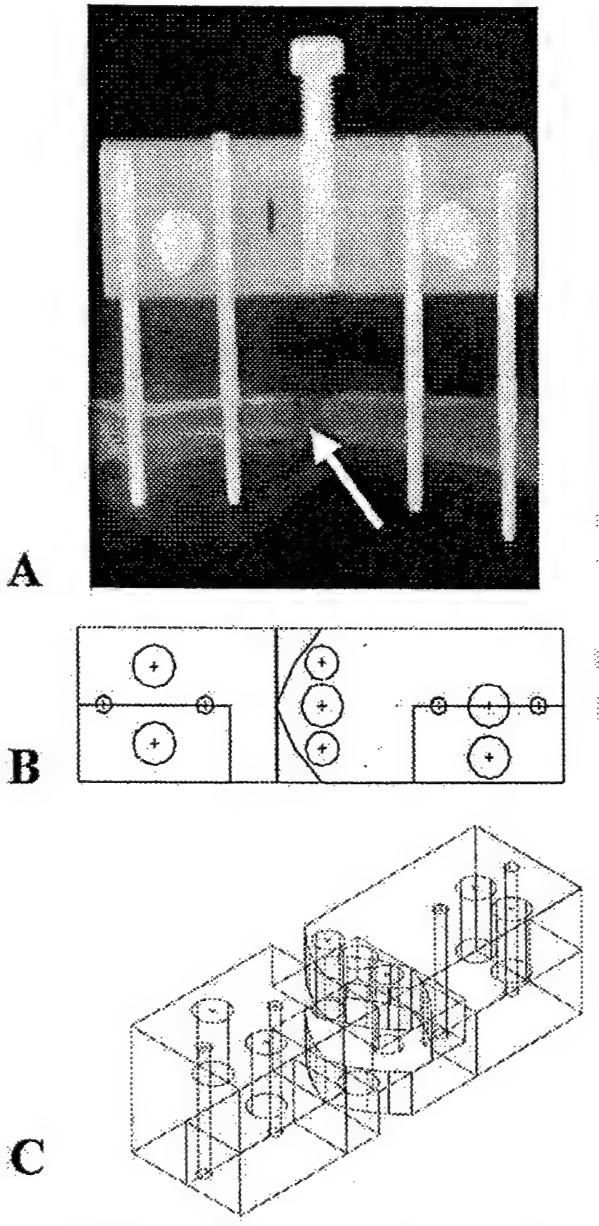

Fig. 1. Radiograph of the fixator and 3.0 mm defect on a rat femur (A) with an Autocad drawing of the fixator in lateral view (B) and obliquely in 3D (C). An arcade can be seen developing within the defect as a result of the micromotion bending treatment (arrow). The action of the fixator in (A) is in and out of the paper. In (B) the pins would be positioned in the small holes along the length of the small rectangles. Note the axis of the fixator is aligned with the theoretical center of the defect.

Materials and methods

Operative procedure

Animal care and experimental protocols were followed in accordance with NIH guidelines and approved by our institution's Laboratory Animal Science Center IACUC. Following a minimum 48 h acclimation period, sixteen skeletally mature male Sprague Dawley rats (400 ± 20 g) were transported to a dedicated surgical procedure room. Each animal was anesthetized with an isofluorene inhalation system (4.0% induction, 2.0% maintenance). The right thigh was shaved, prepped with betadine, and given an intramuscular (all injections were given via this route) injection of Cefazolin antibiotic (200 mg/kg).

A 2 cm incision was made on the lateral aspect of the thigh, creating a plane through the lateral intermuscular septum to expose the femur. The periosteum was elevated on the anterolateral aspect of the femur and a drill guide template was positioned on the femur using two cable ties. Special care was taken in placing the template to allow the pins to be inserted centrally on the diaphysis of the femur, reducing the chances of fracture due to inappropriate pin insertion. Once the template was securely placed, the proximal-most pinhole was drilled using a drill guide and a 0.7 mm drill bit. The hole was tapped with a 0.9 mm tap and the pin was secured into the femur. The other pins were placed using the same technique in order from the distal-most pin to the inner proximal and inner distal pins. The template was removed and the skin pulled over the pins by placing fourteen gauge needle heads over the pin locations. The fixator was secured to the pins as close to the skin as possible without potentially causing skin ulcerations.

Following the application of the fixator, a defect was made using a rotating saw blade attached to a Dremmel Tool (Dremmel, Racine, WI). A 3 mm gap size was chosen so that it would function as a non-critical sized defect allowing healing with delayed union to occur [15]. After completion of the defect, the site was irrigated with sterile saline and the fascia and skin closed. The incision was cleansed with betadine and the animal given an injection of Buprenex analgesic (0.2 mg/kg). During the 2 days following surgery each animal received an injection of Cefazolin (200 mg/kg) once per day, and an injection of Buprenex (0.2 mg/kg) twice per day. Each animal was housed in its own individual cage, and for the duration of the experiment all animals were monitored daily for pain and infection.

Fixator design and application

A custom designed external fixator was used to introduce 40% (vertical displacement as a function of the cortex diameter) interfragmentary bending strain (12° angular excursion) with a hypothetical symmetrical bending load distribution centered within the gap (Fig. 1). It was felt that 12° was well within the physiological range of angular excursion of normal developing joints such as the knee or elbow. To perform this motion an oscillating linkage was built such that the amount of interfragmentary displacement could be set between 1.0 and 1.5 mm relative to the locked position, providing for the 12° of total angular excursion. The motion was applied using a stepper motor (model #2602-010, Hurst Motors, Detroit, MI). The speed of the motor was controlled using an EPC-013 stepper motor control (Hurst Motors, Detroit, MI). The shaft of the stepper motor was coupled to one of two eccentric shafts of a torque transducer (model #1102-50, Lebow Products, Troy, MI). The other eccentric shaft of the transducer was connected to the linkage. A torque sensor connected to an external data acquisition board (model #100, Instrunet, Cambridge, MA) acted as a bridge voltage sensor and controlled the torque transduced to the fixator. Based on manufacturer's specifications the torque sensor was mapped to 1.967 mV/oz in., with the input torque sampled at 60 Hz for the duration of each experimental session. The peak torque required to induce motion was recorded as the baseline value. The loading apparatus was then calibrated prior to every application using Fourier transforms of the stepper motor moment application.

Each of the sixteen animals had an external fixator applied to its right femur and a 3 mm defect created at the mid-diaphysis. One group ($n = 8$ animals) represented the controls and remained rigidly fixed for the entire experimental period, receiving no motion treatment. The treatment group ($n = 8$ animals) received mechanical stimulation at 1.0 Hz for 10 min a day, with a 3 days on, one day off loading protocol for the first two weeks. The loading protocol for the final three weeks was 2 days on and 2 days off. The loading protocol was designed to give the animals weekly rest time from the anesthesia. The treatment frequency of 1.0 Hz was chosen because it falls well within the physiological range of normal joint function. A total of 600 symmetrical bending cycles per day were induced on the defect of the treatment animals for a total of 35 days (24 days of actual stimulation). The experimental time period of five weeks was determined to be the point of bony bridging under rigid fixation based upon the results of the control specimens.

Radiological assessment

High resolution radiographs (Ultraspeed DF-50, Kodak, Rochester, NY) of the harvested specimens were performed weekly until the termination of the experiment. Radiographs were taken in order to monitor fixator stability and placement, and to follow callus mineralization and bony healing.

Histological assessment

Animals were euthanized at day 35, the femora were excised and specimens placed in 4.0% paraformaldehyde for 12 h. They were then decalcified for two weeks in TBD-2 decalcifying solution (Shannon, Baltimore, MD). The bones were then dehydrated via a series of increasing alcohol solutions and cleared in xylene for 1 h. They were infiltrated with paraffin, with the solution changed at 1 and 2 h, and maintained at 60 °C under a 25 mm Hg vacuum. The bones were embedded so as to expose the sagittal plane for sectioning. Specimens were sectioned at 5–7 μm thickness and stained with hematoxylin and eosin, or alcian blue. Immunohistochemical staining for Type II collagen was performed using a primary antibody of Collagen II (Neomarkers, Fremont, CA) and a Vector Stain Kit (Vector Laboratories, Burlingame, CA). Articular cartilage specimens were obtained from the knees of cohort rats.

Fourier transform analysis

Histological slides were utilized to quantify preferred fiber angle and fiber angle conformity in the collagen fibers of the newly formed cartilage tissues (Fig. 2). These values were determined for deep, intermediate, and superficial layers and were compared between the treatment group, the controls, and normal rat knee articular cartilage. First, standard H & E histologic sections of demineralized treatment, control, and normal rat knee cartilage specimens were prepared and collagen fiber orientation within the tissues was graphically visualized under polarized light with an Olympus OMT2 microscope at 200× magnification. Two deep, two intermediate, and two superficial fields were generated and mapped for each of the 16 cartilage surfaces (8 distal and 8 proximal). The superficial zone was determined as the area

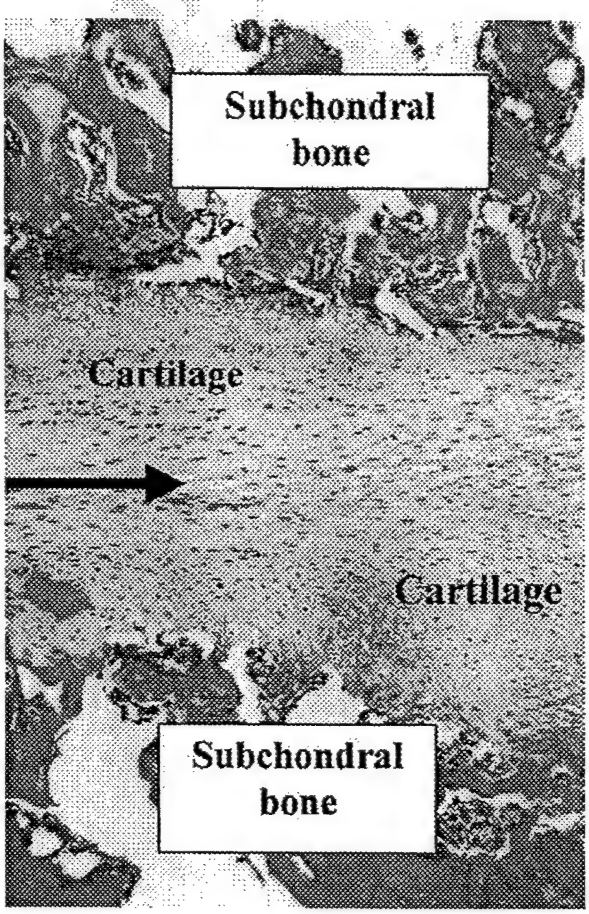

Fig. 2. Cartilage tissue formed in a *neoarthrosis* via controlled micromotion (from Fig. 4(D)). Red tissue at top and bottom are distal and proximal subchondral bone arcades. The morphology of the cartilage cells changes from the arcades to the midline, becoming more flattened. A distinctive cleavage can be seen beginning between the distal and proximal cartilage bands (arrow). It is believed that the introduction of shear micromotionwould further enhance the cleavage of this cartilage zone into two discontiguous cartilage surfaces.

immediately under the cartilage surface, the deep zone was the area immediately adjacent to the underlying subchondral bone, and the intermediate zone was exactly half way between the deep and superficial zones. The polarized microscopic images were captured using a digital camera (Kodak DCS-420, Kodak, Rochester, NY) affixed to the microscope. The digital images were imported into Matlab and thresholds were set to obtain high contrast images (Fig. 3(A)). Preferred fiber orientation within each field was determined by creating Fourier transforms of the digitized images (Matlab, Mathworks, Natick, MA), which generate an elliptical signal (Fig. 3(B)). The gray level of each pixel was processed using a 2D fast Fourier transform $F(m,n)$, where m and n are spatial frequencies corresponding to the x and y axes of the original image. The gray level of each pixel, represented by a function, $f(m,n)$, was then transformed using a 2D discrete Fourier transform.

$$\mathbf{F}(\mathbf{p},\mathbf{q}) = \sum_{m=0}^{M-1}\sum_{n=0}^{N-1} f(m,n)e^{-j(2\pi/M)pm}e^{-j(2\pi/N)qn},$$

$$p = 0,1,\ldots,M-1, \quad q = 0,1,\ldots,N-1.$$

The preferred orientation in the original image was represented by a peak in the power spectrum ($|F(m,n)|^2$, a 2D array, (Fig. 3(C))). To quantify the orientation its intensity was determined as the angular distribution of the power spectrum. The angular distribution was computed as the average of the power spectrum within a narrow fan-shaped region. The largest peak of the intensity of orientation was perpendicular to the preferred orientation of the fibers. Collagen fiber conformity was determined by the ratio of the major and minor axes of the power spectrum peak, with larger values corresponding to greater angle conformity.

Statistical analyses

Data are presented as mean ± S.D. All histomorphometric results including collagen preferred fiber angle and fiber angle conformity were compared between the control and treatment groups using an ANOVA and Tukey's post hoc test at an level of 0.05, and with p values less than 0.05 interpreted as significant. All sample sizes for the specific groups were determined by power statistics calculations based on a coefficient of variation of 25% in the data, and accepting α and β errors of 5.0%.

Results

All six specimens of the control group demonstrated at least partial bony union, with two showing complete union (Fig. 4). Radiological and histological analyses of the treatment group showed the creation of an organized pseudoarthrosis with opposing bone arcade structures capping the defect termini (Figs. 4 and 5). Additionally, a cartilage tissue with articular cartilage-like organization developed on the defect side of the bone arcades (Fig. 2). This complex structure was maintained in the mechanical stimulation group past the time of healing in the controls, with the shape of the arcades corresponding to the pivoting action of the fixator. No control animals demonstrated development of a bony arcade or cartilaginous band across the medullary canal. These arcades, especially when present on both sides (Fig. 4(B)–(D)), had the effect of segmenting the femur into two bones via a joint-like structure. This *neoarthrosis* was maintained in the treatment group past the time of healing in the controls. The tissue arising from the arcades stained positively for sulfated proteoglycan specific alcian blue staining, an indication of the presence of cartilage. Likewise, type II collagen was prevalent throughout the tissue, confirming it to be cartilage. However, the cartilage band was not thick enough to quantitatively determine a trend in staining intensity, as is normally found in articular cartilage. The presence of other specific cartilage types (i.e., Type IX, Type X, Type XI, etc.) was not determined in this study.

The distribution and orientation of collagen fibers within the experimental cartilage closely resembled that of collagen in articular cartilage from rat knee joints, with an alignment parallel to the articulating surface in the superficial zone and an increasingly perpendicular alignment, relative to the articulating surface, in the intermediate and deeper zones (Table 1). The control cartilage (residual from the endochondral osteogenesis process of healing), was fragmented in its distribution within the defect and demonstrated no superficial-to-deep polarity in the level of organization. The greatest similarity in collagen fiber angle was found between the treatment ($4.47° \pm 2.65$) and knee articular cartilage ($4.5° \pm 4.69$) in the superficial zone ($p = 0.89$). The values from the control superficial zone

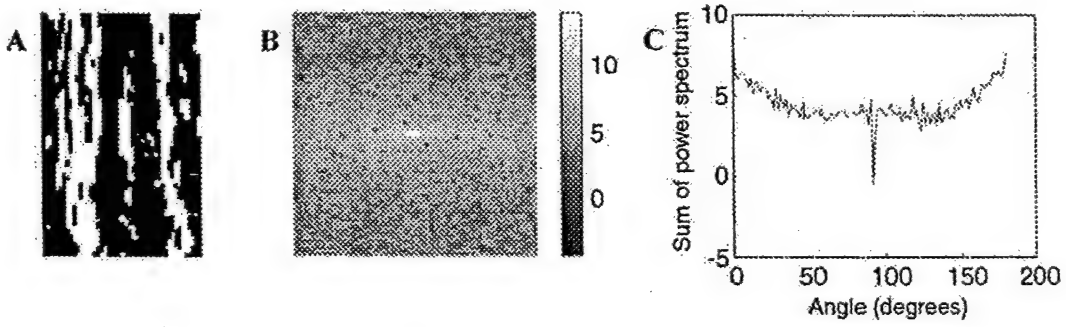

Fig. 3. Stages and results of Fourier transform of polarized neoarthrotic collagen fiber images. (A) Thresholded polarized image of collagen fibers in the superficial zone of the cartilage. (B) Elliptical transform of image in 3A with horizontal major axis and (C) power spectrum analysis of fiber angle preference (90°). The ratio of the major axis to the minor axis in the ellipse in (B) determines fiber angle conformity. Higher ratios denote higher fiber conformity to preferred angle.

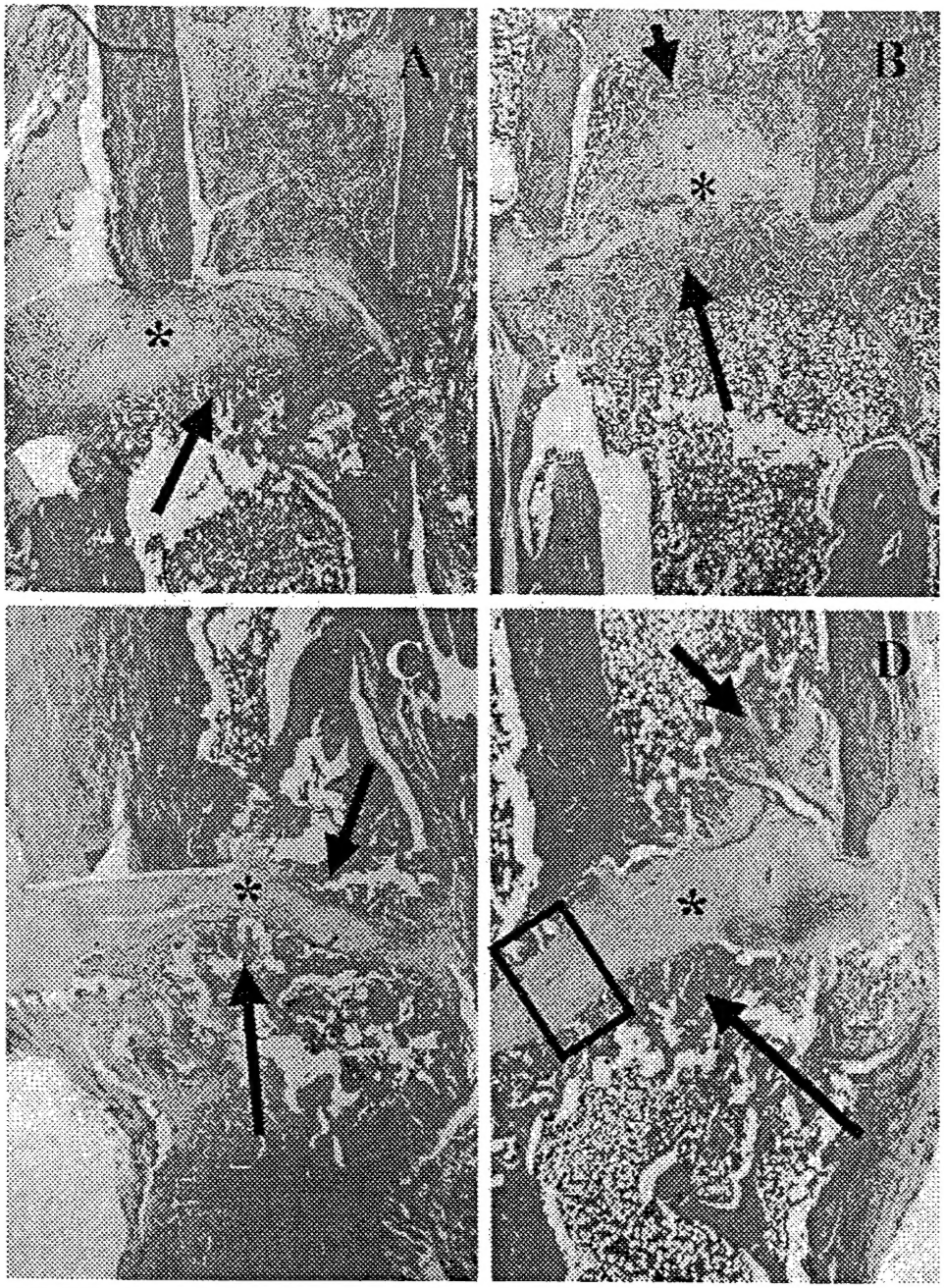

Fig. 4. Bony arcades (arrows) clearly formed in four out of six treatment animals as a result of the controlled micromotion. The bony arcade develops as an extension of the cortices and the convex formation is sometimes matched by a corresponding concave formation on the other side of the defect (B, C, D). The arcades support an articular-like cartilage tissue on the defect side (asterisk). The boxed in area (D) shows the articular-like cartilage forming on the subchondral bone, and at higher magnifications a cleavage line can be seen between distinct cartilage bands (Fig. 2).

(101.67° ± 105.7) were significantly different from the articular cartilage ($p < 0.01$) and treatment groups ($p = 0.04$). In the intermediate zone the knee articular cartilage and treatment groups were not significantly different (7.1° ± 4.62 and 6.55° ± 4.9, respectively, $p = 0.83$). The collagen in the intermediate zone of the control group (35.89° ± 12.72) demonstrated a somewhat perpendicular fiber angle relative to a hypothetical articular surface at the midpoint of the defect. There was a significant difference between the articular cartilage and the controls ($p < 0.01$), and between the neoarthrotic cartilage and the controls ($p < 0.01$). The deep layer in all three groups demonstrated perpendicular preferred collagen fiber angles (relative to the articular

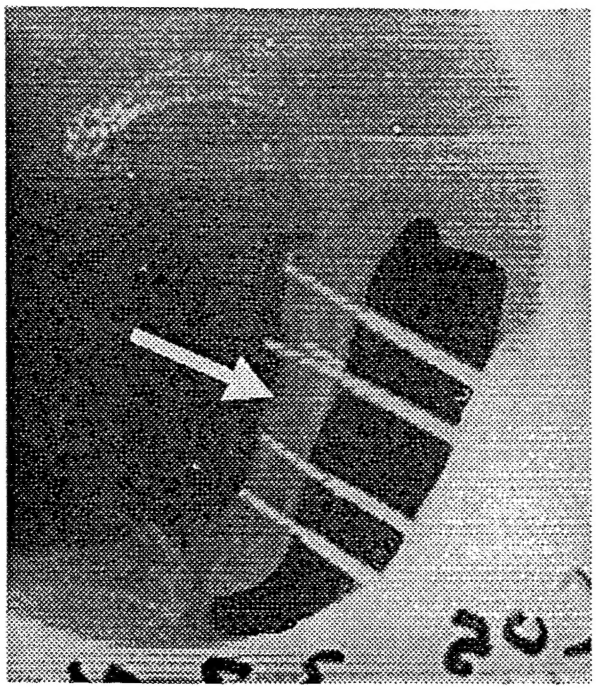

Fig. 5. Histological section of a control specimen demonstrating bony bridging of the defect via direct periosteal bone formation (arrow). Note the lack of endochondral bone formation within the defect space which is the result of ideally rigid fixation (asterisks). Two control specimens demonstrated bony healing while four showed delayed union. No control specimen developed a bony arcade or articular-like cartilage as seen in the treatment group (Fig. 4).

surface), with no significant differences between the three groups.

The conformity ratios (angular agreement of the individual collagen fibers) were highest in the superficial zones of both the knee articular cartilage and the treatment cartilage (6.88 ± 3.83 and 4.04 ± 0.45, respectively, $p = 0.16$) while the control group demonstrated relatively low fiber organization (1.49 ± 0.11). However, a significant difference was found between both the articular and neoarthrotic fiber angle conformities versus the control conformity ($p = 0.02$ and $p < 0.01$, respectively). The only significant difference in fiber conformity in the intermediate zone was found between the articular cartilage and the control groups ($p < 0.01$). There were no significant differences in fiber conformity between the groups in the deep zone. In summary, the collagen within the neoarthrotic cartilage appeared to not be statistically different from that seen in rat knee articular cartilage, while the control cartilage was statistically different.

Discussion

It is well known that humans are capable of forming a cartilaginous joint-like structure in a long bone, as is observed in the pathological condition pseudoarthrosis [12]. Although the precise mechanical and biological events which lead to this condition are not known, theoretical modeling of tissue development within a fracture site predict that such an outcome is possible [2,4,6,9,18,27]. Experimental models of pseudoarthroses have even been established using manually applied mechanical stimulation [1], but precisely controlled mechanical treatments and their effects on tissue differentiation have not been established.

Our results suggest that the application of precisely controlled bending motion in a non-critical sized defect leads to the directed formation of specific tissues such as cartilage (as specified by theoretical models), and that the architectural organization of the resulting anatomical structures relate directly to the action and magnitude of the induced motion. Thus, the resulting pseudoarthrotic structures were likely a response to the symmetrical bending motion, as are the analogous structures in in

Table 1
Results of the collagen fiber preferred orientation and conformity analyses using Fourier transforms of polarized histological specimens

		Superficial layer		Intermediate layer		Deep layer	
		Angle (°)	Ratio	Angle (°)	Ratio	Angle (°)	Ratio
Articular cartilage	Mean	4.5α	6.88α	7.1α	4.87α	64.1	2.97
	S.D.	4.69	3.83	4.62	1.34	31.71	1.39
Neo arthrosis	Mean	4.47β	4.04β	6.55β	3.33	53.2	1.83
	S.D.	2.65	0.45	4.9	1.16	70.09	0.56
Healing controls	Mean	101.67αβ	1.49αβ	35.89αβ	2.16α	76.65	2.32
	S.D.	105.76	0.11	12.72	0.35	82.24	1.29

Preferred fiber angle is relative to the articulating surface except in the healing controls which were referenced to a homologous line perpendicular to the long axis of the bone and through the center of the osteotomy. The fiber angle conformity ratio is the similarity of each individual fiber angle to the average angle, with higher values indicating greater conformity. A striking similarity existed between the neoarthrotic cartilage (Neo Arthrosis) and rat knee articular cartilage (Articular Cartilage) in the intermediate and superficial layers. These were in contrast to the endochondral cartilage found in typical healing fractures such as that represented by the controls. Fiber angle conformity was relatively low in the controls and the standard deviation of the mean preferred fiber angle was very high. Significant differences are denoted as (α) for articular cartilage and (β) for neoarthrotic cartilage.

utero joint development largely a product of their mechanical environment [3,5,13,22,23]. This cyclical and symmetrical bending motion created a mechanical environment of alternating hydrostatic compression and tension in one axis, symmetrically increasing in magnitude with increased distance from the center of the defect to the outer cortex. A neutral axis with respect to tension and compression was created along the theoretical bending axis of the defect. Additionally, the initiation of a line of cavitation between the newly developing surfaces resulted from the shear component of the bending action (Fig. 2, see arrow).

The bone arcades that formed across the termini of the defect effectively capped the segments and created what could be considered two bones out of the single femur. This segmenting of a single skeletal element is a crucial event in joint formation. The bone caps were likely the result of the forces induced by the alternating compressive loads caused by the fixator, compressing the material and cells recruited to repair the defect. In some instances clearly reciprocal convex and concave shaped arcades formed from this bone; likely a result of the pivoting action of the fixator. The arcades that developed were aligned with the fixator bending axis and as the fixator pivoted on its axis so did the healing defect.

The cartilage tissue that developed on these bony caps demonstrated an organized collagen fiber architecture (with respect to the perpendicular of the long axis of the bone segments). Likewise, collagen fiber architecture in articular cartilage is well organized showing superficial to deep polarity [10,26]. In contrast, collagen fiber architecture in cartilage of the endochondral ossification pathway is typically much less organized, having little to no deep-to-superficial polarity in its structure. Although not identical to articular cartilage, the ultrastructure of the cartilage that developed on the bony termini of the experimental group demonstrated a degree of organization which was strikingly similar.

A shear component was also induced on the surface of the defect tissues as they developed, another result of the pivoting action imposed by the fixator. As the tissue initially began to differentiate, the plane of shearing action defined the dividing point between the segments. As the tissues further differentiated into bone and cartilage, the shear component was likely responsible for the specialized orientation of the collagen fibers within the superficial layer of cartilage. Shear forces would be greatest superficially at the opposing surfaces (the location of greatest shear displacement during bending), and be reduced in magnitude proximally and distally from the defect center. Thus, shear would be lower in magnitude in the deeper cartilage tissues where the collagen fiber orientation was increasingly less tangential to the presumptive surface, and deeper still where subchondral bone formed. This is analogous to the mechanical environment responsible for articular cartilage formation in utero.

Studies of the influence of the mechanical environment on fracture healing suggest that less rigid fixation leads to preferred endochondral osteogenesis while more rigid fixation leads to preferred intramembranous ossification [2–4,9,11,18,27]. Our stimulation protocol provided motion, but in precisely controlled directions, followed by 23 h of rigid fixation. We believe the defect segmentation, bone and cartilage differentiation and cartilage architecture are all the result of the controlled mechanical stimulation imposed by the fixator. This is analogous to the case for articular cartilage developing in utero, whose structure results largely from its loading history during development [3,5,13,22,23]. The results of this study indicate that precise control of the mechanical environment during bone defect healing can influence differentiation at both the tissue (cartilage versus bone) and molecular (organized versus disorganized collagen fiber architecture) levels. The significance of this study lies in the parallels of defect healing and in utero joint development [17], and in its potential use in directed tissue differentiation for joint repair.

Uncited reference

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